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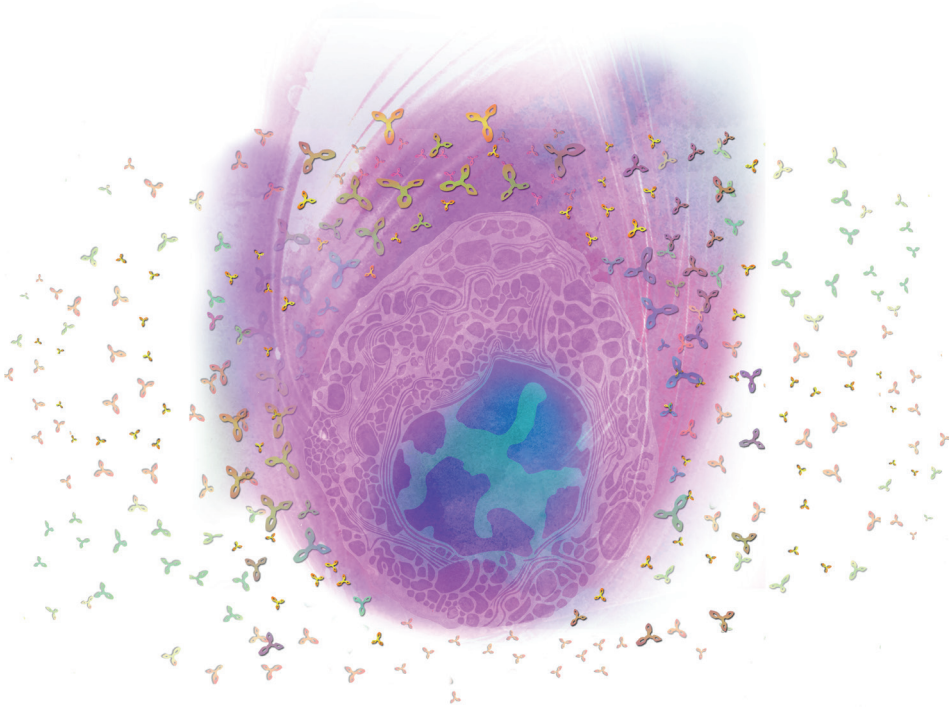
B cell fate following immunization: from memory B cells to plasma cells



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Cover illustration shows a schematic representation of a EM plasma cell and the antibodies released from it by David Ricardo Cadena Martínez, all rights reserved ©

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En homonaje a todas las mujres de mi familia a las que la educación les fue negada, su valor y persistencia me dieron la oportunidad de ser.

ABSTRACT

Most approved successful human vaccines induce neutralizing antibody titers maintained above a given threshold for long-term protection against potential pathogen exposure. The pathogens targeted by these vaccines are antigenically stable and the relevant epitopes are immunogenic. In contrast, HIV-1 displays an enormous diversity in the circulating virus population and in each infected individual, in which the most relevant neutralizing epitopes are poorly exposed and thus less immunogenic HIV-1 is highly prone to immune escape, posing an extreme challenge for vaccine development. Elicitation of antibodies capable of neutralizing a broad range of HIV-1 strains and persist over time are likely to be required for an effective vaccine. This has focused the attention in the field on vaccine-elicited B cell responses against the HIV-1 envelope glycoproteins (Env), the only virally encoded target for neutralizing antibodies. Recent progresses in the design of soluble Env trimers that mimic the native HIV-1 spike have increased the interest in understanding vaccine-induced neutralizing antibody responses. In addition, the durability of vaccine-induced responses is poorly understood. Thus, a better understanding of how to modulate Env-induced responses by using different immunogens, immunization regimens and adjuvants is needed. In this thesis, I used rhesus macaques to investigate several of these questions.

In **Paper I**, we used an early generation Env trimer to evaluate whether the addition of a TLR-9 agonist to Matrix-M adjuvant would impact Env-specific immune responses. We demonstrated that the addition of the TLR-9 agonist had no measurable impact on the kinetics or durability of the B cell response, nor on the peripheral T cell response, the plasma neutralizing antibody activity or the control of viremia after challenge. In **Paper II**, we evaluated antibody responses elicited by new generation well-ordered HIV-1 trimers administered as soluble protein or conjugated to liposomes for multivalent display, both in the presence of Matrix-M adjuvant. We found that liposome-display resulted in superior germinal center (GC) responses and significantly improved neutralizing antibody activity compared to the soluble trimers. We then isolated monoclonal antibodies mediating autologous tier 2 virus neutralizing activity and demonstrated that these antibodies target the Env trimer apex using a lateral binding approach. In **Paper III**, we investigated plasma cell frequencies in the bone marrow (BM). Specifically, we evaluated whether longitudinal BM sampling would affect the frequency of total plasma cells in this compartment. We found this not to be the case; rather we observed intrinsic animal variation and that the frequency of plasma cells correlated with the age of the animals. In **Paper IV**, we described cell markers that better characterize BM plasma cells. We found that functional BM plasma cells that constitutively secrete IgG, IgA and IgM were double positive for CD138 and CD31. These markers allowed the distinction between bone marrow and peripheral plasma cells.

In conclusion, this thesis offers new information about several aspects of HIV-1 Env-induced B cell responses of direct relevance for vaccine development. This thesis also establishes methodology that can be used to further investigate vaccine-induced B cell responses, including in the BM compartment.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. **Martinez P***, Sundling C*, O'Dell S, Mascola J.R, Wyatt R.T, Karlsson Hedestam G.B. Primate immune responses to HIV-1 Env formulated in the saponin-based adjuvant AbISCO-100 in the presence or absence of TLR9 co-stimulation. Scientific Reports. 2015, 5 (8925): 1-11.

II. **Martinez-Murillo P***, Tran K*, Guenaga J, Lindgren G, Àdori M, Feng Y, Phad G.E, Vázquez Bernat N, Bale S, Ingale J, Dubrovskaya V, O'Dell S, Pramanik L, Spångberg M, Corcoran M, Loré K, Mascola J.R, Wyatt R.T and Karlsson Hedestam G.B. Accepted for publication in Immunity.

III. Spångberg M, **Martinez P**, Fredlund H, Karlsson Hedestam G.B, Sundling C. A simple and safe technique for longitudinal bone marrow aspiration in cynomolgus and rhesus macaques. Journal of Immunological Methods. 2014, 408: 137-141.

IV. **Martinez-Murillo P**, Pramanik L, Sundling C, Hultenby K, Wretenberg P, Spångberg M, Karlsson Hedestam G.B. CD138 and CD31 double-positive cells comprise the functional antibody-secreting plasma cell compartment in primate bone marrow. Frontiers in Immunology. 2016, 7 (242): 1-10.

*Equal contribution

The following publications were also obtained during the course of the PhD studies but were not including in this thesis:

- Sundling, C, **Martinez, P**, Soldemo, M, Spångberg, M, Bengtsson, K.L, Stertman, L, Forsell, M.N, Karlsson Hedestam, G.B. Immunization of macaques with soluble HIV type 1 and influenza virus envelope glycoproteins results in a similarly rapid contraction of peripheral B-cell responses after boosting. The Journal of infectious diseases. 2013, 207: 426-431.

- Phad, G.E, Vázquez Bernat, N, Feng, Y, Ingale, J, **Martinez Murillo, P.A**, O'Dell, S, Li, Y, Mascola, J.R, Sundling, C, Wyatt, R.T, Karlsson Hedestam, G.B. Diverse antibody genetic and recognition properties revealed following HIV-1 envelope glycoprotein immunization. Journal of immunology. 2015, 194: 5903-5914.

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
ADCC	Ab-dependent cellular cytoxicity
ADCP	Ab-dependent cellular phagocytosis
Ag	Antigen
AID	Activation-induced cytidine deaminase
aNAbs	Autologous neutralizing antibody
APC	Antigen presenting cell
BCR	B cell receptor
bNAbs	Broadly neutralizing Abs
bp	Base pair
CD4i	CD4-induced
CDRs	Complementary determining regions
CTL	Cytotoxic T lymphocyte
CoRbs	Co-receptor binding site
DAMPs	Damage associated molecular patterns
DCs	Dendritic cells
DC	Deoxycytidine
Du	Deoxyuridine
Env	HIV-1 Envelope glycoproteins
FcR	Fc receptor
FDCs	Follicular dendritic cells
FR	Framework regions
GC	Germinal center
HBV	Hepatitis B virus
HCDR3	Long heavy-chain complementarity-determining region 3
HIV	Human immunodeficiency virus type 1

HPV	Human papilloma virus
Ig	Immunoglobulin
ISCOMs	Immunostimulatory complexes
LLPCs	Long-lived plasma cells
mAbs	Monoclonal Abs
MALT	Mucosa-associated lymphoid tissue
memB	Memory B cells
MPER	Membrane-proximal region of gp41
MSH2	Mismatch repair heterodimers
MPL	Monophosphoryl lipid A
NFL	Native flexible linker
NHP	Non-human primates
nNAbs	Non-neutralizing antibodies
NHEJ	Non-homologous end-joining
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PCs	Plasma cells
pDCs	Plasmacytoid dendritic cells
RAG	Recombination-activating genes
RSS	Recombination signal sequence
RT	Reverse transcriptase
S1PR1	Sphingosine-1-phosphate receptor 1
SHM	Somatic hypermutation
TD	Trimer-derived
TdT	Terminal deoxynucleotidyl transferase
Tfh	T follicular helper cells
Th1	T helper 1

TLRs	Toll-like receptors
UNG	Uracil DNA glycosylate

1 AIMS

The specific aims for the four individual papers were:

Paper I: To evaluate whether the addition of a TLR-9 agonist impacts B cell or T cell responses elicited in rhesus macaques immunized with HIV-1 Env trimers formulated in Matrix-M adjuvant.

Paper II: To determine whether particulate display of HIV-1 Env trimers on liposomes offers an advantage over soluble trimers for elicitation of neutralizing antibody responses in rhesus macaques.

Paper III: To evaluate the impact of longitudinal bone marrow sampling on plasma cell frequencies in rhesus macaques.

Paper IV: To develop improved methods to isolate functional bone marrow plasma cells from rhesus macaques by flow cytometry.

2 VACCINES

2.1 VACCINE PROGRESS

Vaccination as a practice has its origins in the 18th century, when the physician Edward Jenner and the farmer Benjamin Jesty observed that cowpox-exposed milkmaids were unaffected by smallpox and inferred that cowpox infection protected them against smallpox. This wise observation guided Jenner to conduct what can be considered a vaccine clinical trial, after which he confirmed that inoculation of blisters from cowpox-exposed milkmaids into healthy individuals protected them against subsequent infection with smallpox (reviewed in [1]). In the 19th century Louis Pasteur made another essential observation in the field of vaccinology; he observed reduction in the virulence of the causative organism of chicken cholera by altering the method of culturing it. Pasteur coined the concept of *in vitro* or *in vivo* attenuation, approaches that were subsequently used in his work with vaccines against anthrax and rabies.

At the beginning of the 20th century it was well known that genetic selection for avirulent strains could be achieved by the passage of the organism in an atypical host. Subsequently this enabled the *in vitro* attenuation of bacteria (e.g. Mycobacterim bovis bacille Calmette-Guérin), and the *in vivo* attenuation of viruses (e.g. yellow fever virus) to develop vaccines. However, it was not until the latter part of the 20th century that vaccine development made a major advance, thanks to a methodological breakthrough that took place in 1949: the capacity to grow viruses in cell culture. The controlled growth of viruses allowed the successful development of whole virus-based vaccines against many diseases such as polio “Sabin”, measles, mumps, rubella and varicella zoster (attenuated live vaccines) and polio ”Salk”, tick borne encephalitis, rabies, hepatitis A (HAV) and seasonal influenza (Flu) virus (inactivated vaccines). The live attenuated virus vaccine concept would also become the basis for the vaccinia virus that was used as a vaccine against smallpox, which in 1980 led to smallpox being the first virus that was globally eradicated through vaccination [1-3].

During the 1980’s the vaccine field would move towards the development of subcomponent vaccines, which are safer in immunocompromised individuals because they use only parts of the infectious agent in the vaccine preparation. Some of the subcomponent vaccines include crude preparation extracts against flu, anthrax or rabies, toxoids against tetanus and diphtheria, bacterial capsular polysaccharides conjugation to toxoids against meningococci and pneumococci. Other vaccines are based on well-defined genetically engineered, recombinant proteins assembled into virus-like particles, such as against hepatitis B virus (HBV) and human papilloma virus (HPV). In the case of the HPV vaccine, genes encoding the L1 proteins of oncogenic serotypes were expressed using yeast or insect expressiongardasil systems for production of virus-like particles [4, 5].

While the vaccine field was developing improved methods for production of vaccine components, knowledge of the immune system also increased. It became clear that an

improved understanding of the basis for protective immunological responses would be required for successful vaccine development against many pathogens.

2.2 ANTIBODIES AS THE MAIN MEDIATORS OF EFFECTIVE PROTECTION

Since 1950's, it has been known that the presence of antibodies (Abs) against a virus correlates with immunological protection in most individuals. The measurement of the specific immune response (type and levels of Abs and T cell responses) has been essential to define the effector functions that are responsible for protection, referred as the correlate. However, it is not always possible to identify a correlate. In some cases the measurement of an immune response parameter that correlates with protection, but which is not proven to mediate protection, is used instead and is denoted a surrogate [6, 7]. For example, in zoster vaccination antibody and cellular responses are induced and correlate with efficacy of the vaccine, therefore both are correlates; while, cellular response had a statistically stronger correlation, which in light of the biology of the disease indicates that the cellular response is a mechanistic correlate, while the antibody response is a surrogate [7]. The identification of markers of correlation (correlates or surrogates) with protection against infection, or disease after vaccination (or natural infection), is important because it guides the choice of antigens and adjuvants to be included in the vaccine and it helps to define immunity at individual and population levels [6].

Most successful vaccines against low rate mutation viral pathogens induce protection in the population by the combination of two different mechanisms: preventing infection or enabling faster recovery from infection. To prevent infection, the vaccine should induce persisting Abs and memory B cells that will increase production of Abs at local and systemic levels; while the T cell responses are important to contain replication by eliminating infected cells. As shown following measles vaccination, Abs can protect against both disease and infection when neutralization titers reach >1000mIU. However, children with T cell-deficiency may suffer complications from the vaccine as a result of the lack of functional T cells necessary to contain viral replication even though the virus is attenuated [6]. Correlates or surrogates have been defined for many invariant viral vaccines, and in general Abs have been shown to play a predominant role in protection. However, due to the high redundancy that characterizes the immune system, multiple immune responses interact to protect the host, and although B cell memory is crucial for prolonged protection after vaccination, it is dependent on the magnitude of the innate immune response that enhances adaptive cellular responses when these are established following immunization. Additionally, there are many other factors to be taken into account when it comes to eliciting an effective Ab response: localization of the Abs (site of pathogen replication); breadth of the response to affect heterologous serotypes; specificity and durability of the response and immune status of the vaccinated individual [6, 8].

However, correlates or surrogates are not well defined for pathogens with complex life cycles or rapid mutation rates, such as malaria or HIV-1 since effective vaccines have yet to be developed. It is believed that they may include a combination of vaccine-induced cellular and

humoral immune responses [9]. Efforts to develop an HIV-1 vaccine have therefore stimulated the field to study platforms that induce both T and B cells responses, as well as new adjuvants and immunogen delivery strategies.

2.3 FEATURES OF A PROTECTIVE ANTIBODY RESPONSE

As mentioned above, the smallpox vaccine led to the complete eradication of this infectious disease, therefore is considered a vaccine gold standard. In this section I will review some of the immunological properties that are important for vaccine-induced protection.

2.3.1 Specificity and neutralization capacity of the response

Although most of the currently approved human vaccines mediate their protection through antibodies, our knowledge about the specific target epitopes they recognize in the different approved vaccines is poor and mainly derives from evaluation of serum and plasma responses. The identification of specific B cell epitopes is important to improve our understanding of protective immune responses to vaccination. This level of resolution requires the isolation of monoclonal Abs (mAbs) from vaccinated individuals; a task that is labor intensive and therefore is rarely performed if the vaccine is considered to be effective.

However, when vaccine candidates do not induce the desired protection, as for the HIV-1 immunogens tested so far, mAb isolation provides concrete information about the fine specificities of the response. Such studies are valuable to understand the limitations of the response and to guide further vaccine design efforts [10, 11]. Some Env determinants are highly immunogenic such as variable region 3 (V3) [12, 13]. So far, HIV-1 Env immunization elicits a range of specificities, which are either non-neutralizing or which neutralize Tier 1 viruses (laboratory-passaged strains or primary strains that are unusually easy to neutralize) [10, 14-17]. Neutralization of Tier 2 strains, which are representative of circulating HIV-1 isolates, has proven to be considerably more challenging in well-validated neutralization assays [18, 19]. Occasional heterologous Tier 2 neutralization has been reported with assays that are now considered insufficiently stringent, such as the A3R5 assay [20]. In contrast, autologous Tier 2 neutralization can be achieved by vaccination [21, 22]; Paper I), but the specificities mediating these activities are only now beginning to be understood ([23]; Paper II)). A better understanding of vaccine-induced neutralizing Ab specificities is essential to accelerate the development of a vaccine against HIV-1 with the aim to target more conserved epitopes.

2.3.2 Breadth of the response

In the cases where successful vaccines have been developed, it is because the target pathogen is antigenically stable and the relevant epitope regions are immunogenic. For Flu, several prominent strains may circulate each year and once these are defined, it is possible to develop a seasonal vaccine. In contrast, for HIV-1 there is enormous diversity in the circulating virus population at all times, making vaccine design an extreme challenge (reviewed in [24-28].

The study of mAbs isolated from individuals chronically infected with HIV-1 has identified several epitopes on Env that are target of neutralization. From some individuals, it has been possible to isolate broadly neutralizing Abs (bNAbs) that can neutralize a wide range of neutralization resistant (Tier 2) strains [29-39]. Many bNAbs have characteristic features such as high levels of somatic hypermutation (SHM), long heavy-chain complementarity-determining region 3 (HCDR3) regions and restricted germline use such as for the VRC01-class of bNAbs directed to the CD4-binding site [34, 40]. However, presently no immunogen has been able to induce such antibodies in a host with a natural immune repertoire.

Based on what we know so far, immunization with the seasonal influenza vaccine, recombinant HIV-1 Env or tetanus antigens induce polyclonal Abs with similar SHM levels [10, 41, 42]; Paper II). In the cases where this has been studied, the elicited Abs do not appear to increase in SHM or affinity much after a couple of boosts, indicating that they have reached an affinity ceiling, as suggested by Foote and Eisen in 1995 [43]. The levels of SHM elicited through conventional vaccine strategies are not sufficient to generate the high levels of mutation observed in most bNAbs isolated from HIV-1 infection (sometime more than 30%). To reach such levels would require multiple rounds of selection in the germinal center in response to a continuously evolving pathogen, such as HIV-1. To promote increased levels of SHM, novel vaccine strategies are needed, such as regimens involving heterologous boosting using different immunogens or different vaccine delivery platforms as performed in the field over the past several years [19, 44], an approach that is now intensely explored for germline-targeting immunogens [45-47].

2.3.3 Durability of the response

Current knowledge supports the view that plasma cells residing in the bone marrow are responsible for the maintenance of long-lasting serum Ab responses induced by infection or vaccination [48]. The duration of serum antibody responses has been estimated for several of the current human approved vaccines, with half-lives ranging from 50 years for varicella–zoster virus to more than 200 years for other viruses such as measles and mumps [49]. In the case of the smallpox vaccine, the estimated half-life of the serum-specific Ab response was 92 years while the Ag-specific memory B cell response was maintained for more than 50 years after vaccination. The frequency of Ag-specific memory B cells correlated with serum-specific Ab levels and also showed a similar kinetics with an initial decline followed by decades of apparent stability [49, 50].

Although investigating long-term antibody responses is important in the development of effective vaccines, it requires decades to formally demonstrate the durability of a protective response. We have established baseline information about the kinetics of Env-induced Ab responses in peripheral blood and in the bone marrow of immunized non-human primates. Peripheral Ab and memory B cell-specific Env responses follow the same kinetics, where they are detected after the first immunization while the peak is reached after the second immunization. In absence of further boosting there is then a relatively rapid contraction in the peripheral response. The half-life of the Env specific-IgG response is 21 days, which suggest

that these Abs are secreted by short-lived plasmablasts generated from the antigen-specific memory B cells that expanded and differentiated into short-lived plasmablast after boosting [17, 51]; Paper I and II). However, there is a low level of Env specific-IgG that persist during the long-term boost, these Abs are though to generate from PCs archived in the BM, where also low levels of Env specific-IgG PCs have been reported [17, 51]; Paper I).

2.3.4 Protective threshold

Optimally, a successful vaccine should elicit long-lasting Abs capable of cross-reacting with diverse strains of the pathogen. In addition, the Abs should reach above a certain threshold to elicit their protective effect. The smallpox vaccine, for example, confers protection through neutralizing antibodies, where the protective titer needs to be ≥20 neutralizing units [6]. Passive immunization studies with anti-HIV-1 Abs have been performed in macaques to assess the bNAb concentration required to prevent infection by chimeric simian/human immunodeficiency viruses (SHIVs) [52-54]. These studies showed that protection was related to the antibody concentration and half-life in serum and the protection required serum antibody concentrations corresponding to the IC80 in TZM-bl neutralization assay [52].

Some groups of people, like infants, elderly and immunocompromised individuals, have difficulty reaching the protective threshold with current vaccines. In addition, the vaccine response starts to decline in healthy adults at 40-50 years of age. Therefore, the addition of immunostimulatory substances called adjuvants, which can potentiate the response to reach protective titers, has been introduced for some vaccines targeting these groups, such as the influenza vaccine. The introduction of adjuvants in the influenza vaccine has resulted in improved immune responses in these groups [55]. As the vaccine field is moving more and more toward recombinant protein-based vaccines, which are highly pure and therefore lack intrinsic adjuvant effects, it is increasingly important to identify safe and effective adjuvants that can be used in humans.

2.4 ADJUVANTS

The need of safe and effective adjuvants that can be used with recombinant protein-based vaccines has driven a renewed interest in this field. Currently, only few adjuvants are approved for human used and others are in various stages of clinical development, with some main examples described below.

2.4.1 Purpose of an adjuvant

As mentioned above, vaccine development focuses increasingly on reductionist approaches with well-defined subcomponents to better control the elicited immune response and improves safety profiles. Although this approach increases antigen purity in comparison to inactivated and attenuated vaccines, it also reduces immunogenicity. This has made it necessary to use adjuvants to boost the adaptive immune response towards the antigen. The enhancement in adaptive immune response can be qualitative or quantitative, and different adjuvants are under development for this purpose.

Quantitative improvement of vaccines through adjuvants aims to 1) increase antibody titers and consequently the number of subjects that become protected in the general population; 2) increase seroconversion rates in poorly responsive populations; and 3) reduce the vaccine dose. While qualitative improvements aim to promote different arms of the immune system, such as 1) functionally appropriate types of T helper 1 (Th1) cell and Th2 cell responses; 2) CD8+ T cell responses; 3) different antibody isotypes; 4) increase the speed of the initial response; and 5) alter the breadth, specificity, or affinity of the response [56].

In the case of HIV-1 vaccine development, the use of an adjuvant capable of promoting broad and potent Ab responses is desirable to, in the best case, prevent infection, or, more likely, help clear the virus and dampen the acute viremia after potential exposure. Furthermore, simultaneous induction of balanced CD4 and CD8 T cell responses, as well as Ab-dependent cellular cytoxicity (ADCC) activity are desirable to contain viruses that may escape Ab mediated neutralization.

2.4.2 Modulation of immune responses by adjuvants

The adaptive immune system has evolved in the presence of the innate immune system and they are fully integrated. This is reflected by the expression of multiple innate recognition receptors on T and B cells that enable them to directly interact with the innate immune system. Innate immune cells have the ability to recognize pathogens directly or indirectly; directly, through the recognition of molecular structures present on pathogens (pathogen-associated molecular patterns or PAMPs) by pattern recognition receptors (PRR) encoded by the host germline or indirectly through the recognition of damage associated molecular patterns (DAMPs), such as ATP or uric acid, released upon tissue damage [57, 58].

Adjuvants are typically classified into immunostimulatory agents, or passive depots or vehicles. Innate immune stimulation is achieved through the ligation of PRR family members that include Toll-like receptors (TLRs), NOD-like, RIG-I-like and C-type lectin receptors. PRR ligation induces downstream signal activation to activate transcriptional programs involving the production of cytokines, chemokines, and co-stimulatory molecules that play key roles in priming, expanding and polarizing immune responses (reviewed in [59, 60]).

2.4.3 Adjuvants approved in humans

2.4.3.1 Alum

The first adjuvant to be approved for human vaccination was aluminum-based salts, commonly referred to as alum. Alum has been highly successful due to its safety profile and its ability to enhance antibody responses. Since its discovery in 1926 by Alexander Glenny, it was accepted that the adjuvant activity was due to its ability to promote antigen persistence by providing a depot effect. However, recent interest in developing new adjuvants has renewed interest in investigating the mechanism of action of alum [61]. Some reports suggest that alum activates the NLPR3 inflammasome pathway directly (phagocyte engulfment of alum) and indirectly (release of endogenous DAMPS such as uric acid) to promote antibody

production [62, 63], while others have shown that Alum-induced antibody responses are independent of NLRP3 [61, 64]. Thus, this question remains controversial.

2.4.3.2 AS04

AS04 is one of a series of adjuvants developed by GlaxoSmithKline (GSK). In brief, it is a combination of aluminum salts and the LPS derivative monophosphoryl lipid A (MPL) [65]. Two licensed vaccines use AS04, FENDrix® and Cervarix®, which confer protective immunity against HBV [66] and HPV [4], respectively. AS04 induces a transient localized innate immune response and activation of dendritic cells (DCs) that stimulate antigen-specific T cells, mainly CD4+ T-cells and antigen-specific Ab responses. MPL modulates the quality of the immune response towards a balanced Th1/Th2 response [67, 68].

2.4.3.3 MF59

Emulsion adjuvants exist in two forms: "water-in-oil", when aqueous droplets form the emulsion dispersed within an oily media, and "oil-in-water", where the opposite is formulated. MF59 is an oil-in-water emulsion based on squalene, a precursor molecule of natural cholesterol that is more easily metabolized. MF59 was developed by Novartis, which now is a part of GSK [55]. It is well tolerated in humans and has been licensed for use in an influenza vaccine targeting the elderly population (Fluad®). MF59 induces local immune stimulation with recruitment of DCs and granulocytes promoting influenza-specific CD4+ T-cell responses and Ab responses [69]. The mechanism of action of MF59 has been reported to be independent of the NLRP3 inflammasome [60].

2.4.4 Adjuvants in development

2.4.4.1 Matrix-M and Iscomatrix

The Matrix-M and Iscomatrix adjuvants form cage-like nanoparticles (around 40nm in diameter) composed of a mix of purified saponin fractions, extracted from the South American tree, Quillaja saponaria, mixed at specific ratios with cholesterol and phospholipids. Over the years, the Matrix formulations have been optimized to minimize reactogenicity while maintaining adjuvant effect. Matrix-M^{TM} (Novavax) was previously referred as Abisco-100 when it was distributed by Isconova. Matrix-M consists of two well-characterized saponin fractions, fraction-C with potent adjuvant activity and fraction-A with weaker adjuvant activity. By mixing these two fractions at a specific ratio, potent adjuvant activity with high safety profile was achieved [70]. A similar adjuvant, Iscomatrix, was distributed by the Australian vaccine company CSL [71]. These adjuvants do not act through any identified PRR. Instead, it is thought that the immune stimulation is achieved through endosomal stress and eventual apoptosis of tissue-resident antigen presenting cells (APCs) that have taken up and internalized the adjuvant. This event initiates infiltration of monocytes and neutrophils that subsequently transport the antigen to draining lymph nodes for T- and B-cell activation. In the lymph node, the adjuvant promotes an increase in pro-inflammatory cytokines (IL-5, IL-8, IFN-γ) and cellular trafficking with activation and prolonged retention

of DCs. This environment promotes effective activation of the adaptive immune response, resulting in potent Ab production, a balanced Th1/Th2 CD4 T cells and even some induction of CD8+ T cell responses [70, 72-74]. Matrix-M has now been tested in several clinical flu vaccine trials [75, 76] to name just a few of the vaccines where Matrix-M is under evaluation.

2.4.4.2 TLR-9 agonists

The best understood family of PRRs is the Toll-like receptors (TLRs), they were first described as homologues of the Toll-receptor in Drosophila melanogaster [77, 78]. They and are evolutionary highly conserved and in humans consist of ten different subtypes (reviewed in [79]). TLRs are transmembrane PRRs that can recognize PAMPs both extracellularly and in endosomal compartments. Those expressed at the cell surface primarily recognize extracellular PAMPs, such as bacterial membrane components, where for example TLR4 recognizes lipopolysaccharide (LPS), while those localized in endosomes, such as TLR9, detect nucleic acids. This localization allows efficient detection of invading viral and bacterial DNA via sensing of unmethylated CpG dinucleotides, these motifs are less common in vertebrate DNA, preventing accidental stimulation by CpG motifs within self DNA [80]. In humans, TLR9 is expressed in plasmacytoid DCs (pDCs) and B cells [81, 82]. In Paper I, of this thesis we evaluate the effect of adding a TLR-9 agonist to HIV-1 Env trimers formulated in Matrix-M.

Synthetic oligodeoxynucleotides (ODN) with unmethylated CpG dinucleotides (CpG ODN) mimic the immunostimulatory activity of bacterial or viral DNA and activate TLR9 *in vitro* and *in vivo*. Synthetic CpG agonists have been used in attempts to enhance immune responses in vaccine studies. CpG ODN can be classified into three groups based on their distinct chemical and biophysical properties, as well as immunomodulatory activity: CpG-A, consists of palindromic unmethylated DNA sequences that form aggregates. They have a phosphodiester chemical backbone with phosphorothioate G-rich ends. CpG-A is a strong activator of pDCs with resulting high levels of interferon alpha (IFN-α) production. CpG-B does not contain palindromic sequences, remains soluble, has a phosphorothioate backbone, and is a potent B cell stimulator. CpG-C combines properties of both CpG-A and CpG-B. It has a phosphorothioate backbone but no poly-G stretches. There are, however, palindromic sequences and stimulatory CpG motifs that strongly stimulate B-cell, pDC and natural killer (NK) cell activation with IFN-α production [83, 84]. Rhesus macaques are a good model to monitor activity and safety of CpG ODNs *in vivo*, due to their capacity to recognize and respond to the same motifs that activate human immune cells [85]. However, there are differences in the responses induced in human and macaques after TLR9 agonist stimulation. For instance CpG-C induce more potent B-cell proliferation in rhesus peripheral blood mononuclear cells (PBMC) cultures than CpG-A or CpG-B, while CpG-B stimulates more potent B-cell proliferation in human cultures [86].

3 ANTIBODY DIVERSITY AND LONG-TERM HUMORAL MEMORY

The notion of Abs has been around since the late 1800s, when von Behring and Kitasato reported the important role of circulating antitoxins against diphtheria and tetanus. Subsequently, Ehrlich proposed a cellular source for Abs (reviewed in [87]). However, it was not until 1948 that Fagreaus demonstrated the cellular origin of antibodies, when she found a correlation between the plasma cell development and Ab titers after immunization [88]. Later, the distinction between T- and B-cell lineages was reported by Max Cooper [89] and since then, the description of the fine details that govern the diversity and production of Abs has been a central interest in immunology.

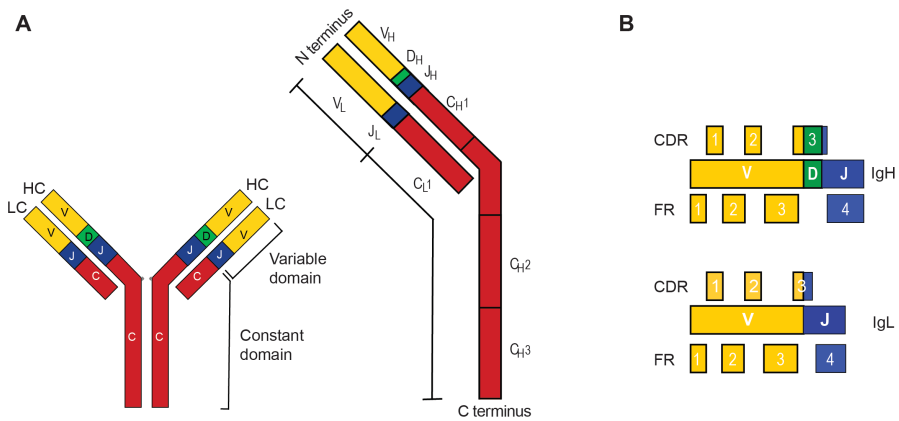

Figure 1. IgG antibody structure. (A) The structure of an antibody consists of two heavy chains (HC) and two light chains (LC). Each chain is composed of a variable domain and a constant domain (left). The variable domain of HC is encoded by one V, one D and one J gene segment, while the LC is encoded by one V and one J segment (right). **(B)** Each variable domain is composed of three regions referred to as complementarity determining regions (CDR) 1-3 and four framework (FR) regions within the VDJ and VJ segments. The most variable region of an antibody is the HCDR3 that covers the end of the V segment, the entire D segment and the first part of the J segment with non-templated nucleotides inserted between the segments. *Illustration by Pierre-Yves Mantel.*

3.1 ANTIBODY STRUCTURE AND GENETICS

Studies on the physical nature of Abs first took place in the 1930s, when Tiselius and Kabat used electrophoresis to separate immunized serum into albumin, α-globulin, β-globulin, and γ-globulin fractions. Absorption of the serum against the antigen depleted the g-globulin fraction, yielding the terms γ-globulin and eventually the term immunoglobulin (Ig) was coined. In most mammals, Igs consist of two heavy (H) and two light (L) chains. The L-chain can consist of either κ or λ, which are encoded by different genetic loci. Enzymatic treatment of Abs with papain led to the description of two fragments: a homogeneous H-chain fragment that allows crystallization (Fragment crystallizable, Fc) and a non-homogeneous H-chain portion that remains attached to the L-chain and retains the binding to the antigen (Fragment

of antigen binding, Fab). The C-terminus domain of both H and L chains defines the isotype and effector function of the Ab, while the N-terminal domain interacts directly with the antigen and is responsible for the Ab specificity. The N-terminal variable domain consists of three hypervariable regions, referred to as complementary determining regions (CDRs) and four conserved regions, termed framework regions (FR) (reviewed in [90]) (see Figure 1).

3.1.1 Mechanisms of antibody diversity

The humoral immune system has evolved the capacity to respond to and neutralize almost any pathogen that the host may encounter through the production of Abs. Abs are the secreted form of the membrane bound B cell receptor (BCR). The potential pre-immune Ab repertoire is estimated to consist of over 10^{16} different immunoglobulins that are generated through rearrangement of germline sequences during B-cell development [91]. The actual functional B-cell repertoire is likely not quite as great as not all rearrangements are productive. Furthermore, many B cells that have a functional BCR still get removed during development due to the generation of a self-reactive BCR. This process is referred to as central tolerance. The serum repertoire of IgG has been estimated to consist of over 10^4 unique antibodies, while the peripheral memory B cell repertoire is three or more orders of magnitude higher [92]. Of course there may be caveats with these estimations due to technical limitations of the methods used to generate these numbers.

3.1.1.1 Rearrangement of gene segments

The rearrangement of germline gene segments is one of the main mechanisms to diversify the Ab repertoire. The rearrangement takes place during B cell development in the omentum, fetal liver or adult bone marrow and is used to produce a primary repertoire of low affinity antibodies, prior to encounter with foreign antigens. The very sophisticated mechanism of sequential rearrangements of individual Ig gene segments, which generates the variable domain of the BCR was first described by Tonegawa in 1983 [91]. The H- and L-chain loci have multiple variable (V), diversity (D) and joining (J) gene segments. H-chain has V, D, and J segments that rearrange first, whereas the variable domain of L-chain consists of V and J gene segments (Figure 2).

The recombination events require a mechanism that ensures correct joining of the V(D)J gene segments. This is achieved by enzymes targeting specific DNA motifs, the recombination signal sequence (RSS). RSS motifs are short sequences that flank V(D)J segments, and consist of heptamer or nonamer sequences separated by a 12- or 23-base pair (bp) spacer, corresponding to one or two turns of the DNA helix. In the H-chain locus, the RSS is located 3-prime to each V gene segment, 5-prime to each J gene segment and on both sides of each D gene segment. RSS having one-turn spacer can join only with sequences having a two-turn spacer (the so called one-turn/two-turn joining rule). This joining rule ensures that segments of the same type do not join each other, and also ensures that the segments join in proper order (reviewed in [90]).

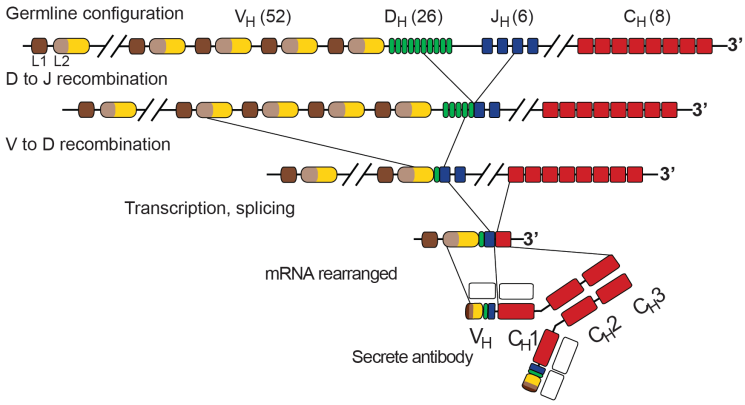

Figure 2. Schematic representation of VDJ rearrangement of the human HC locus. The recombination events that take place in the immunoglobulin loci are mediated by two enzymes Rag-1 and Rag-2, expressed mainly during lymphocyte development. In pro-B cells, Rag proteins first bind the RSS motifs at the 3'end of the D segment and the 5' of the J target segments to mediate recombination. Rag then targets the 3' of the V segment and the 5´of the already rearranged DJ segments, resulting in a VDJ segment. In pro-B cells, splicing events at RNA level mediate the assembly of the already rearranged VDJ segment with a gene segment of the constant domain to encode the variable region of a functional antibody. *Illustration by Pierre-Yves Mantel*

Initiation of the V(D)J recombination reaction takes place in early pro B cells and requires recombination-activating genes (RAG) 1 and 2, which are expressed almost exclusively in developing lymphocytes. RAG1 and RAG2 bind the RSS at the heptamer and induce a DNA single-strand break between the end of the gene segment and its adjacent heptamer, resulting in the reaction of hydroxyl groups with the uncut strand and the formation of closed hairpin ends. The hairpin is then cleaved at a random site by a complex of endonucleases (Artemis). The cut ends of the coding sequence are then repaired by the non-homologous end-joining (NHEJ) proteins. This process creates precise joins between the RSS ends and imprecise joins of the coding end, which results in short palindromes, known as P-nucleotides. Terminal deoxynucleotidyl transferase (TdT) adds N-nucleotides randomly to the single stranded ends forming the junctions, thereby contributing to the diversity of the CDR3, which spans the V(D)J junctions. Unpaired nucleotides are trimmed by an exonuclease and the coding joint is repaired (Figure 2) (reviewed in [90]).

The size of the theoretical primary repertoire varies from species to species depending on the number of germline V, D, and J segments. For instance, in humans 52, 26 and 6 functional H-chain V, D and J gen segments respectively, have been reported [93], while in rhesus macaques 50 (V), 39 (D) and 6 (J) [94]. Sequence analysis of human and rhesus macaques has shown that the V-genes cluster according to VH family distribution rather than according to species, indicating the high level of gene family conservation between these two primate species [10] as well as indicating inter VH-family expansion over evolutionary time. The diversity of the primary germline repertoire relies on the combinatorial diversity of the germline V, D, and J gene segments, as well as on the junctional diversity conferred from

imprecision at the sites of V-D-J gene integration, where deletion or non-templated insertion of nucleotides takes place adding to the diversity.

3.1.1.1 Somatic hypermutation (SHM)

A second mechanism that increases the diversity of the antibody repertoire is somatic hypermutation (SHM). SHM is based on random point mutations introduced primarily in the V(D)J regions by the enzyme activation-induced cytidine deaminase (AID), which is upregulated in proliferating B cells exposed to cognate antigen in the presence of T cell help [95, 96].

SHM induces single nucleotide substitutions in V(D)J genes, beginning around 150 nucleotides down-stream of the transcription initiation site. It reaches a peak and declines over about 2 kb, which focuses mutations to the rearranged V(D)J segment. AID activity correlates with transcription activity and it functions by changing deoxycytidine (dC) into deoxyuridine (dU), transforming C:G pairs into U:G mismatches that initiates a repair program. There are at least three mechanisms of repair with different outcomes (reviewed in [95]):

-Introduction of adenine (A), that takes place when the U is read as a T during cell division and DNA replication.

-Insertion of any nucleotide (A, T, G, C) after the uracil DNA glycosylate (UNG) excises U during DNA replication or DNA repair.

-Single-stranded sequence gaps generated by mismatch repair heterodimers MSH2 and MSH6 that are then repaired by error prone DNA polymerases

3.2 ACTIVATION OF ANTIGEN-SPECIFIC B CELL RESPONSES

Antibody production results from a B-cell differentiation process that starts when the BCR on a mature naïve B-cell binds to an antigen. BCR signaling prompts migration of the B-cell to the border of the T-cell zone, where it receives signals from activated T cells. Through these signals, each naïve B-cell receives cues to proliferate and differentiate into one of four types of effector cells: short-lived plasma cells, germinal center (GC) cells, GC-independent memory cells and undifferentiated activated precursors [97, 98].

3.2.1 B cell activation

Each naïve B-cell expresses a unique BCR on the cell surface that is capable of recognizing a cognate antigen. The naïve B cells circulate through secondary lymphoid organs where they encounter antigen brought there primarily by lymphatic drainage. Naïve B cells can also bind soluble antigens that diffuse from the sinus into the follicles, as well as particulate antigens that accumulate on the surface of subcapsular sinus resident macrophages and paracortex DCs [99-101]. For many years it was considered that in the absence of antigen the BCR on naïve cells was able to freely diffuse in the cell membrane in a conformation that does not

allow oligomerization, and that upon antigen binding, the BCR would adopt an oligomerization-permissive conformation, that allows oligomerization and formation of microclusters with other antigen bound BCRs [102, 103]. However, recent findings suggest that the BCR on naïve cells is already forming independent pre-clusters of IgD-BCRs and IgM-BCRs (protein islands), that upon activation move in closer proximity to each other, affecting subsequent signaling events [104].

The signaling of the BCR initiates the formation of an immune synapse. Upon activation, the naïve B cells quickly contract the synapse and extract the antigen for endocytosis and degradation into peptides to be loaded onto major histocompatibility complex class II molecules (MHCII) [105], while they upregulate the expression of CCR7 and EBI-2, which together guide migration to the edge of the follicle bordering the T cell area [106]. At the follicular border, activated B cells present the peptides to activated cognate CD4+ T cells. This interaction induces the expression of CD40L and secretion of cytokines (IL-4 and IFN-γ) from the T cells [107]. Stimulation of CD40 and cytokine receptors induces B cell proliferation in the outer follicle; 12–24 h after activation some of these primary (naïve) as well as secondary B cells can undergo class switch recombination and further differentiation into one of four fates, in part, according to their affinity for the antigen [108]. Activated B cells with higher affinity differentiate into short-lived plasma cells that migrate to the extrafollicular area under EBI-2 influence [106], where they secrete low-affinity antigen-specific germline encoded Abs that are important for early protective Ab responses; while activated B cells with low affinity, primarily differentiate into GC B cells or extra-follicular memory B cells (memB) [109, 110].

3.2.2 Germinal center reaction

GCs were first described in the late 19th century and were thought to be responsible for the production of new lymphocytes. They were established as part of the immune response (reaction to the entry of foreign antigens) early in the 20th century. Later, key features, such as complement-dependent localization of antigen on the follicular DCs (FDCs), T-cell dependence, predominance of B cells and de novo SHM were established. Currently, and in agreement with Burnet's clonal selection theory, it is well accepted that SHM and affinity based selection of antigen-activated B cells takes place mainly within the GC (reviewed in [108]). Therefore, the formation of GCs and its downstream consequences (affinity maturation, isotype switched memory B cells and long-lived plasma cells) is a key feature when investigating novel vaccination strategies.

GCs were first identified as anatomical structures, clearly visible by conventional histology techniques that were divided into dark and light zones (DZ and LZ, respectively) [111]. GCs are very dynamic structures and their formation takes place when activated B cells in the outer follicles re-enter the interfollicular areas, this migration is mediated through the loss of CCR7 and EBI-2, and expression of CXCR5. T follicular helper cells (Tfh) and activated B cells migrate from the interfollicular area towards the center of the follicle, which is rich in FDCs. It is here that activated B cells undergo extensive proliferation that push the resident

follicular B cells aside to form the early GCs. GCs expands quickly as a result of rapid proliferation with the eventual formation of mature GCs, characterized by the LZ and DZ areas. The GC DZ is situated proximal to the T-cell zone, and consists mainly of proliferating B cells that remain there because of a high CXCR4 expression [112]. CXCL13 is a potent chemoattractant produced by FDC, which induce the movement of GC B cells and Tfh towards the LZ through interaction with its receptor CXCR5 [113]. The LZ contains a network of FDC rich in cognate antigen immune-complexes, antigen-specific Tfh and GC B cells. Formation of the GC is dependent on upregulation of the transcription factor Bcl-6 in GC B cells and Tfh cells. Bcl-6 acts as a transcriptional repressor of Blimp-1 to induce and maintain the GC B-cell and Tfh-cell phenotype and survival [114].

The measurement of GC activity is important in the development of novel, more efficacious vaccines. Currently the preferred means of quantifying the GC response is through cellular enumeration and analysis of GC Tfh and GC B cells by flow cytometry and immunofluorescence (IF). Tfh can be identified in mice, humans and primates as $CXCR5^{hi}PD1^{hi}Bcl6^{hi}$ CD4 T cells that secrete CXCL-13, IL-21 and IL-4. In mice GC B cells can be identified by their expression of high levels of Fas and n-glycolylneuraminic acid (the ligand of antibody GL-7), binding to peanut agglutinin, loss of surface IgD and CD38 [115], whereas in primates they are identified by their high expression of Ki-67 and Bcl-6 [116].

3.2.3 Affinity maturation

The process of affinity maturation of antibodies results from two major mechanisms that take place in the GC: generation of a repertoire of mutated clonal variants and the positive selection of high affinity clones. This results in the progressive increase of serum Ab affinity over time. Positive selection of high affinity clonal variants takes place in the LZ and requires the presence of antigen-immune complexes on FDCs. Tfh cells then provide further helper signals to the activated B cells and license them for migration to the DZ, where they divide and mutate. Therefore, for affinity maturation to occur, GC B cells must dynamically migrate between these two zones.

3.2.3.1 Positive selection of high affinity clones

In the LZ 10–30% of B cells are selected to re-enter the DZ to experience further rounds of proliferation while the remainder either die by apoptosis or exit the GC [117, 118]. This selection is based on positive signals available to GC B cells in the LZ, such as BCR signaling and cognate Tfh interaction but also negative signals, such as Ab feedback inhibition [115]. BCR stimulus alone is not enough to induce positive selection, as exogenously delivered antigen increase the death rate [108]. Furthermore, GC B cells have surface BCR densities that are, on average, 10-fold lower than those on naive and memory B cells. This may facilitate more stringent affinity-based selection of somatically mutated variants [109]. Recently, studies have found that GC B cells are unresponsive to BCR signaling mediated by antigens in solution, whereas high affinity antigens bound to plasma membrane sheets can efficiently trigger strong proximal BCR signaling of GC B cells.

However, these signals show poor propagation to activate NF-κβ, which is suggested to require further input from Tfh cells in the form of CD40-ligation [119].

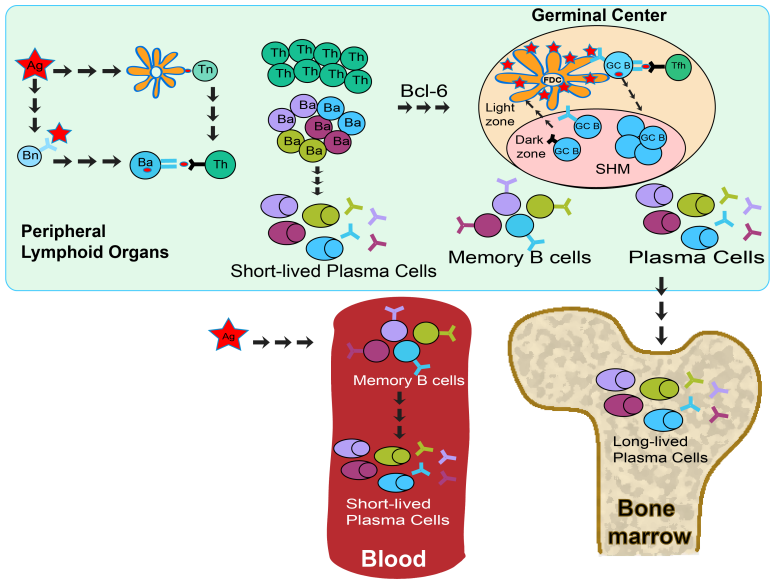

Figure 3. Primary antibody response. During a humoral T-dependent B cell response, naïve B cells (Bn) and naïve T cells (Tn) are activated by antigen (Ag), directly or after processing by an APC. During the prime, activated T cells (Ta) are polarized to one of several T helper (Th) cell types with distinct cytokine profiles. Interaction of activated B cells (Ba) with cognate Th cells induces proliferation of both Th and Ba cells. Proliferating Ba cells can undergo class-switch recombination (CSR) and differentiate into short-lived plasma cells. Ba and Th cells may also up-regulate the transcriptional factor Bcl-6 and establish germinal centers (GCs) where the antibody genes, in particular the V segments, undergo SHM to generate a repertoire of mutated variants that are then subjected to selection according to their affinities. The GCs are divided into two anatomical areas, the light and dark zones, in which cycles of proliferation and SHM in the DZ followed by antigen-driven selection in the LZ result in affinity maturation of the B cell repertoire. In the GCs, T and B cells are distinct populations referred as T follicular helper cells (Tfh) and germinal center B cells (GC B). The end products of the GC reaction are memory B cells and plasma cells. Memory B cells circulate in peripheral tissues and secondary lymphoid organs. Upon encounter with cognate Ag they may differentiate into short-lived plasma cells. In contrast, long-lived plasma cells home into the bone marrow where they receive signals that are essential for their longevity. *Illustration by Paola Martínez.*

The sampling of antigens in the LZ is governed by the unique characteristics of the GC B cell synapse, where antigen is arranged in clusters at the edges of the synapse, and transferred from the APC to the GC B cells, and facilitates contact-mediated repulsion regulated by cytoskeleton components to support continuous antigen sampling in the LZ [119]. This allows efficient retrieval of antigen from the FDC that are processed and presented to cognate Tfh, thus providing a mechanism whereby T cells can sense B cells affinity indirectly through the density of peptide-MHCII complexes on the GC B cells. It is therefore proposed that selection would be driven by competition between GC B cells displaying different surface densities of peptide-MHCII to a limited number of Tfh cells. T-cell help received in the LZ controls re-entry into the DZ and the magnitude also determines the number of division cycles a B-cell can undergo in the DZ. It has been proposed by Victoria *et al* [120] that the

average Tfh helper signal induces two S-phase initiation events in the LZ for each one in the DZ [121], whereas a weak signal results in only one cell cycle per LZ/DZ cycle. This is achieved through a shortening of the cell cycle upon strong T-cell help, allowing multiple cell cycles in the DZ before returning to the LZ (reviewed in [120]).

Antibody feedback can also contribute to GC selection by masking antigen on FDC, thereby effectively reducing available antigen and increasing competition between B cells bearing BCRs of overlapping specificities. Low-affinity clones would be expected to survive if they are specific for epitopes against which little secreted Ab exists, which potentially explains how clones of different affinities can coexist (reviewed in [120]).

3.2.3.2 Clonal diversity

Previously, it was thought that only a few clones would seed each individual GC. However, recent studies using flow cytometric analysis of photoactivated GCs [118, 120] have shown that clonal diversity evolves and is lost at different rates in different GCs. In brief, clonal diversity in early GCs is high (ten to hundreds of different clones) and dependent on the frequency of specific naive precursors or the immunodominance of certain epitopes. In mature GCs B cells of different affinities can co-exist since several clonal variants can undergo affinity maturation without constriction of the clonal diversity. Simultaneously, variants with higher affinity undergo rapid expansion-referred as ‘‘clonal bursts’’- that leads to substantial loss of diversity in a subset of GCs [122, 123]. Based on this, Victora *et al* proposed a model where a stochastic component (any factor not directly related to BCR affinity: fortuitous encounters with antigen-rich FDC clusters or with high-avidity Tfh cells) cooperates with changes in affinity to determine the fate of a mutant GC B cell [120]. In this model, the stochastic factor induces survival signals in B clonal variants that did not improve affinity by SHM, whereas in B clonal variants with an improved affinity a clonal burst will follow [108, 120].

3.2.4 Memory B cells and plasma cells

Long-term effector functions, such as memory and Ab production, rely on the differentiation of B cells into memory B cells (memB) and plasma cells (PCs). For many years it was thought that the generation of memory was a GC-restricted process. However, recent studies have shown that memB can also arise via GC-independent mechanisms [124, 125]. The GC-independent generation of memB cells seems to be important to maintain a broad range of antigen-specific effector B cells that can respond rapidly. It is unclear, however, whether memB generated via a T cell-independent pathway has a functional advantage rather than simply increasing the frequency of antigen-specific cells (reviewed in [126]). The establishment of antigen-specific memBs and PCs are fundamental for long-term immunity and these cells were therefore studied in this thesis.

3.2.4.1 GC-dependent memory B cells and plasma cells fate

Upon positive selection some B cells interrupt the LZ-DZ re-entry cycle to exit the GC. PC differentiation has been associated with higher-affinity, whereas recent results suggest that lower-affinity B cells are preferentially recruited into the memB cell pool [127]. The timing of the humoral response can also influence the B-cell fate choice; memB cells are generated mostly in the pre-GC and early GC periods, while long-lived PC (LLPC) differentiation is more pronounced in later GCs [128]. The question of where GC-derived PCs develop was controversial with some studies suggesting that PC commitment takes place in the LZ due to expression of Blimp1 and IRF-4 initiates there [129]. While other studies have proposed a DZ origin for PCs based on cell migration dynamics [117]. A recent study resolved some of these issues by showing that PC differentiation initiates amongst high affinity LZ GC cells in a BCR-dependent process. These cells then interact with Tfh cells that license them for migration and further differentiation into PCs in the DZ, where they also exit the GC [130].

The molecular mechanisms controlling differentiation to memB cells are unclear. However, a recent study showed that higher expression of the transcriptional repressor Bach2 in lower-affinity GC B cells leads to memB cell differentiation [127]. The PC differentiation program, however, has been better defined, with identification of the key transcription factors Blimp-1, XBP-1 and IRF-4. Blimp1 is the master regulator of PC fate as it helps to shut down expression of the transcription factors Pax-5 and Bcl-6, which are important for B cell and GC lineage commitment [131] and essential for the formation of mature PCs [132]. Low levels of IRF-4 promote GC fate, whereas high levels facilitate PC differentiation by repressing Bcl-6 and activating Blimp-1 and Zbtb-20 [48, 133, 134]. XBP-1 mainly promotes immunoglobulin processing and ER remodeling, which are necessary features of plasma cells with their high levels of antibody secretion [135].

3.2.4.2 Memory B cells

Memory B cells comprise heterogeneous subpopulations that are generated in a spatiotemporal manner and may have different functions. The functional features that define memB cells are their longevity and their rapid and robust response upon antigen re-exposure. The prototypical memB cells have been defined as class-switched B cells with mutated IgV genes and CD27 surface expression (IgG+CD27+). These cells have a GC-dependent origin and localize nearby contracted GCs in secondary lymphoid organs such as spleen to facilitate antigen encounters [136]. Recent studies in mice have reported a new subpopulation of memB cells IgM+ [137] that has also been reported in humans [138]. These IgM+ memB cells mainly originate from GC-dependent responses and share typical functional features with IgG+ memB cell, such as enhanced responsiveness to re-stimulation, metabolism, proliferation, and a propensity for plasmablast differentiation [137]. However, upon T-cell dependent recall responses IgM+ memB cells were shown to be able to re-enter the GC reaction, while IgG+ memB cells mainly differentiate into PCs [110, 138]. IgG+ memB cell kinetics in humans show an initial decline upon resolving infection, followed by decades of apparent stability without the presence of cognate antigen, and correlates with antibody titers

[49, 50]. Kinetics of IgG+ memB cells has been evaluated in rhesus macaques following immunization with HIV-1 Env [51, 139], and the same methods have been applied in this thesis (Paper I).

3.2.4.3 Plasma cells

Astrid Fagreaus identified PCs in the middle of the 20th century, as the cells responsible for Ab production [88]. As mentioned previously, GC independent responses take place outside of the B-cell follicle and generates short-lived PCs, which produce the majority of early protective Abs, characterized as having few mutations with moderate to low affinities. In contrast, PCs generated in GC-dependent responses, appear at late stages of the GC when clones reach high-affinity. These cells then migrate to the BM, where they receive niche-dependent survival signals from stromal cells.

The BM is believed to harbor most of the LLPCs in healthy individuals, which are the main source of long-term circulating Abs. The belief that PCs can survive long-term with sustained antibody secretion derives in part from a study in mice that found 60% of the BM PCs persisting for more than 90 days after antigenic exposure [140]. In addition, studies in humans have reported on vaccine-specific Ab responses with half-lives beyond the lifespan of a human being [49]. Conceptually, lifelong antigen-specific Ab titers can be sustained by the constant generation of short-lived plasma cells from circulating memory B cells [49, 140-144] or by long-lived plasma cells in survival niches [49, 140, 144]. Many questions remain to be answered about long-lived immunity. For example, a recent report in humans showed that the intestine harbors a population of LLPCs, which primarily produce IgA and have specificity for childhood intestinal infections such as rotavirus [145]. This study supports the notion of the PCs can survive for long periods of time in the correct niche and this may not be restricted to the BM.

Homing of PCs to the BM requires efficient egress from the GC. This is mediated through sphingosine-1-phosphate receptor 1 (S1PR1), which drives the cells to enter the bloodstream [146]. CXCR4 expression on PCs and CXCL12 secreted by stromal cells in the BM are then important for the recruitment and retention of the PCs in this compartment [147, 148]. BM PCs in humans can be identified by loss of cell surface expression of CD20 and the high expression of CD138 and CD38. Surface IgG expression is also lost, whereas IgM and IgA plasma cells seem to keep some Ig surface expression [149]. Since BM derived PCs are a critical component of a long-lived vaccine response we investigated these cells in macaques immunized with HIV-1 vaccine candidates (Papers III and IV).

4 HIV-1 HUMORAL RESPONSE

Today, HIV-1 affects over 36 million people worldwide. Since the late 80´s HIV-1 has been a high priority for basic and clinical research and is now one of the main research topics in infectious disease. A tremendous scientific effort has been put into developing effective therapies and vaccine candidates against this highly mutation-prone pathogen. While the development of anti-retroviral drugs has been successful [150], the development of an effective prophylactic vaccine has so far not been accomplished. During the 90s, several clinical trials based on monomeric Env immunogens were conducted, which either failed [151, 152] or demonstrated modest or questionable efficacy [153]. These trials taught the field that a deeper understanding of the Env trimer structure, HIV-1 pathogenesis and host immune responses is necessary to develop an effective vaccine. So far, HIV-1 has broken many paradigms in vaccination and it is clear that new and innovative approaches will be needed to develop a successful vaccine.

Because almost all existing successful vaccines provide protection by their abilities to induce neutralizing antibodies, induction of neutralizing antibodies is also a focus in the HIV-1 vaccine field. However, when it became clear that such responses are very difficult to induce by vaccination parallel efforts to develop a T cell-based vaccine emerged [154, 155]. However, antibody-based vaccines are considered the most important vaccine approach, and the modest levels of reduced risk of infection achieved in the RV144 human vaccine trial has further stimulated this notion.

4.1 HIV-1

HIV-1 was isolated in 1983 and was identified as a member of the Retroviridae family due to the expression of a reverse transcriptase [156]. Further cloning of the genome revealed that it belongs to the Lentivirus genus, which is related to slow disease progression and with lymphocytes as target cells. Retroviruses are enveloped by a lipid membrane, which derives from the infected host cell upon budding. Most enveloped viruses mediate fusion of the viral and host lipid bilayers by changing the conformation of their envelope glycoproteins to form a post-fusion "six-helix bundle" (reviewed in [157]). Understanding the different conformational forms HIV-1 Env can acquire is central to the design of Env-based vaccine candidates.

4.2 ENVELOPE GLYCOPROTEIN

HIV-1 envelope glycoprotein (Env) is the only virus-encoded protein present on the virus surface. Therefore, it is the only viral protein of relevance for the development of an Ab-based vaccine. Env is a trimer of heterodimers consisting of the exterior glycoprotein, gp120 non-covalently associated with the transmembrane protein gp41, whose main function is to mediate viral entry. First, gp120 binds the primary host cell receptor CD4 inducing a conformational change in gp120 that transiently exposes the co-receptor binding site (CoRbs) for interaction with the co-receptor (CCR5 or CXCR4). Binding to the co-receptor stabilizes

the trimer and releases the gp120-mediated hold of the metastable conformation of gp41. This in turn results in structural rearrangements in gp41 domains that liberate enough energy to impulse the insertion of the fusion peptide into the host cell membrane to mediate virus-cell fusion [158, 159].

Env is translated as a gp160 precursor polypeptide that, when synthesized, undergoes signal peptide cleavage, folding, trimerization and extensive glycosylation in the endoplasmic reticulum [160]. The resulting gp160 trimer is transported to the Golgi apparatus where it is cleaved into non-covalently linked gp120 and gp41 subunits by furin-like proteases [161]. Final modifications of the many N-linked glycans also take place in the Golgi apparatus. Thus, three gp120-gp41 protomers assemble into a functional trimer, to form the Env spike. HIV-1 gp120 is composed of five constant regions and five variable regions flanked by conserved cysteines that form loop structures, while gp41 contains a long cytoplasmic tail, a transmembrane domain and a glycosylated ectodomain that is capped by gp120 [162, 163]. The Env spike anchors into the host cell membrane through the hydrophobic membrane-spanning domain of gp41, while gp120 mediates the attachment of the spikes to the target cells through the receptor and co-receptor binding sites [158]. The natural instability of the mature Env spike has substantial implications for the production of soluble Env proteins, as vaccine antigens intended to induce neutralizing Abs must mimic the native trimer. Hence, in the vaccine candidates used in this thesis, multiple modifications were introduced to increase the stability of Env trimer when produced as a soluble glycoprotein. The goal for immunogen design is to create a soluble trimer that is as native-like as possible, which means maintaining bNAb epitopes intact, but reducing the exposure of non-neutralizing Ab epitopes.

4.3 HIV-1 IMMUNE EVASION STRATEGIES

Due to the functional importance of Env, HIV-1 has evolved several strategies to escape Abs-mediated neutralization. These strategies are focused on the selection of Env variants through a Darwinian process in which the most survival-fit variants that confer Ab escape will persist.

4.3.1 Genetic variability

One of the main features of HIV-1 is its high genetic variation that derives from three mechanisms: 1) error-prone HIV-1 reverse transcriptase (RT) that incorporates on average 0.2 mutations per viral genome and replication cycle [164, 165], arising from the high replication rate of 10^{10} new virions per day in an infected individual and high viral load [166]; 2) recombination of viral genomes as a result of super-infection [167, 168] and 3) Apobec-introduced mutations [169]. Immune escape variants are selected from the pool of functional viruses produced in the infected host at any given moment during the chronic infection process. Each new variant represents potential new epitopes for B and T cells. Longitudinal studies have shown that the viral variants and the antibodies against them co-evolve following Darwinian selection theory, in which intense immune selection pressure posed on viral variants results in mutation and selection of variants that are transiently resistant, which

over time results in mutation and selection of broadly neutralizing antibodies in some individuals [170, 171].

4.3.2 Conformational masking

Kwong *et al* described a mechanism of neutralization escape referred as conformational masking in which high affinity recognition by CD4bs Abs requires a conformational change (entropic penalty). This entropic/conformational barrier is imposed on Abs against the CD4bs and the CoRbs, which are the surfaces that undergo most of the conformational changes necessary for the fusion process. The conformational masking is the result of the constriction on Abs orientation imposed by glycosylation and oligomeric occlusion, in combination with the intrinsic flexibility and domain organization of gp120 [172]. Thus, Ab binding to the CD4bs induces a unusually high entropy indicating a thermodynamic mechanism for neutralization resistance typical of non-potent CD4bs Abs, while some CD4bs Abs such as b12 are capable of binding with a small change in entropy [172]. As mentioned earlier, gp120 undergoes a conformational change upon binding CD4 that forms the co-receptor binding site (CoRbs) to allow the high affinity interaction with CCR5 or CXCR4. Although, many Abs are capable of binding the CD4-induced (CD4i) regions, these Abs are generally not capable of neutralizing primary HIV-1 isolates due to steric restrictions [173]. CD4i Abs are abundantly elicited in primates due to the high affinity interaction between Env and host surface CD4 [174].

4.3.3 Evolving glycan shield

HIV-1 Env is a highly glycosylated protein. Approximately, 50% of the gp120 mass is composed of glycans and the sequence contains about 25 potential sites for N-linked glycosylation (PNGs, defined by the amino acid sequence motif NXT/S) [175, 176] or more, depending on the strain. In contrast, other glycosylated viruses such as the influenza virus are not as densely glycosylated. The high density of host-derived glycans covering Env, commonly referred as the glycan shield, has a principal role in immune evasion. It is an evolving and dynamic coat that shifts PNGs and induces viral adaptation in response to intense host immune pressure as an efficient way to generate HIV-1 Ab resistance [177, 178]. Therefore, the model of an evolving glycan shield proposed by Wei *et al* is characterized by the induction of non-glycan-reactive neutralizing Abs that bind contemporary autologous viruses in response to an evolving and plastic glycan shield that prevents binding of neutralizing Abs to the underlying peptide surface.

4.3.4 High immunogenicity of non-native Env

During HIV-1 infection the Ab response generated against Env is dominated by high titers of non-neutralizing antibodies (nNAbs) elicited by non-functional forms of Env, such as monomeric gp120, gp41 stumps, unprocessed gp160 and degradation products of gp120 and gp41 [179, 180]. The non-functional forms of Env result from trimer disintegration (Moore, 2006) as well as from inefficient processing and assembly of spikes [181]. Due to the intrinsic instability of Env, gp41 stumps and shed gp120 appear on the cell/viral surface and

in the blood, respectively. These Env components are highly immunogenic and expose viral surfaces that are not exposed on the native functional Env spike of circulating HIV-1 isolates [182]. It has long been known that nNAbs make up most of the total Env binding titers in plasma from infected [183] and immunized subjects [184], and that neutralizing Abs are just a small fraction of the total Env-directed Ab response. Minimizing exposure of non-neutralizing epitopes on Env-based immunogens is a major focus in immunogen design. It is currently unknown if B cells encoding nNAbs compete with B cells encoding potential bNAb precursors during a polyclonal response [185], or if B-cell responses directed against different epitopes on Env develop independently as suggested by previous studies from our laboratory [186]. Further studies to resolve this issue are needed.

4.4 TYPES OF ANTIBODIES INDUCED DURING HIV-1 INFECTION

As discussed above, Env-specific antibodies elicited during HIV-1 infection can be divided into three major types: 1) Non-neutralizing antibodies (nNAbs), 2) autologous neutralizing (aNAbs) and 3) broadly neutralizing antibodies (bNAbs). Each of these classes is described in more detail below.

4.4.1 Binding but non-neutralizing antibodies (nNAb)

Binding Abs against Env can be detected already 1 week after HIV-1 infection; the first Abs detected, are against gp41 and then towards V1-V2 variable regions of gp120, but due to the lack of neutralization activity these Abs do not induce selection pressure on the virus or affect plasma viral load [187, 188]. As discussed previously, these Abs recognize epitopes on Env determinants that are not exposed on the native Env trimer spike [181, 182, 189]. Because neutralization requires binding to the functional spikes these Abs bind Env but do not have neutralization activity. Despite the lack of neutralization activity, nNAbs could have important antiviral activity through their Fc fragment, which interacts with Fc receptor (FcR)-bearing cells to mediate ADCC and Ab-dependent cellular phagocytosis (ADCP). Although, the presence of nNAbs against V1V2 regions is a correlate with low risk of infection and these Abs mediate ADCC *in vitro*, strong evidence of induction of complete protection against infection as well as complete suppression of viremia *in vivo* is so far lacking [190, 191].

4.4.2 Autologous neutralizing antibodies (aNAb)

Autologous or strain-specific neutralizing Abs, as the name implies, neutralize only the homologous virus that induced them, but not heterologous viruses. aNAb are found in high titers, weeks or months after binding Abs and are detected usually after viremia has decreased (around week 8) [178, 192]. aNAbs are directed against regions that can tolerate a high degree of variability such as Env variable loops, they induce a selective immune pressure in autologous sensitive viruses that drives constant viral evolution through some of the evasion mechanisms described before (section 4.3) such as glycan shifts, direct alteration of an epitope sequence and conformational masking, resulting in Abs that are unable to neutralize the most recently generated viral variant [178, 192-194].

4.4.3 Broadly neutralizing antibodies (bNAb)

The isolation of Abs capable of neutralizing a broad spectrum of HIV-1 primary isolates has offered valuable information. The first set of bNAbs was isolated by using either phage display libraries from PCs or immortalization of B cells. Due to the poor efficiency of these isolation methods only four bNAbs were available for many years: b12, which targets the CD4bs [30], 2F5 and 4E10 that target the membrane-proximal external region (MPER) of gp41 [33, 195], and 2G12 that targets high mannose glycans on gp120 [35]. Before the isolation of bNAb became a standard procedure in many research labs, two main developments took place: identification of HIV-positive donors with bNAb activity in study cohorts by defining broad and potent *in vitro* neutralization activities in plasma samples using well standardized and large viruses panels, as well as new isolation methods such as direct functional screens of single B cell cultures and single B cell sorts of antigen-specific B cells. These technical improvements have allowed the isolation of hundreds of bNAbs from HIV-1 infected individuals, and subsequently to better understand their genetic features and define new target epitopes and evolution pathways (reviewed in [26, 28, 196, 197]).

The isolation of bNAbs from some infected individuals proves an important point: that the human immune system is capable of generating Abs that can neutralize broad panels of HIV-1 strains. However, it takes years to induce Abs with such a great degree of neutralization breadth and potency. These antibodies display particular features not found in other Abs to overcome the specific evasion strategies of HIV-1, such as higher levels of SHM [198], frequent use of insertions and deletions [199], long heavy-CDR3 regions [200], N-glycan recognition [201] and restricted germline use [202].

5 VACCINE STRATEGIES AGAINST HIV

Recombinant monomeric gp120 (AIDVAX) was used in early clinical trials aimed to induce protective Ab responses; however, no efficacy was observed [151, 152]. Later on, the RV144 vaccine clinical trial suggested that a modest protective effect of 31% was achieved using a canarypox virus vector (ALVAC) encoding Env as a prime and a mix of clades B and E gp120 (AIDVAX gp120 B-E) as boosts [153]. Several immune correlate studies have been performed to attempt to understand the effect in the RV144 trial, a correlation between V1V2-directed IgG3 responses and efficacy was found [203]. Furthermore, vaccine efficacy was suggested to be increased when viruses with genetic signatures at two positions in V2 was reported [204].

Over the past several years, most immunogen design efforts have focused on soluble trimeric Env immunogens to attempt to generate variants that more closely mimic the functional spike. The native trimeric Env spike contains multiple bNAb epitopes that are available to the immune system to induce similar specificities. These epitopes are located at the trimer apex, the high-mannose patch, the CD4-binding site, the gp120-gp41 interface and MPER (reviewed in [26, 28, 196, 197]). Therefore, the development of trimer-based immunogens that better mimic the native Env spike is a priority in the HIV field. In the design and development of such Env immunogens, bNAbs and nNAbs are useful tools to probe their three dimensional conformations as well-ordered trimers should be recognized by bNAbs but not by nNAbs. The hope is that such well-ordered trimers will enhance the quality of the induced response (reviewed in [24, 205]).

5.1 IMMUNOGEN DESIGN

The development of trimeric mimics of the Env spike requires a construct that contains both gp120 and gp41 subunits adopting a trimeric conformation. However, the high tendency of Env to transition from a metastable pre-fusion state that maintains the gp41 packed and confined by the association of the gp120 cap into a lower energy post-fusion state represents a major challenge to develop immunogens that mimic the native Env spike.

The three-dimensional configuration of the trimeric Env complex was not elucidated until recently, thanks to the development of new techniques and reagents. For example, negative-stain microscopy (NS-EM) allowed visualization at 20Å resolution to reveal individual subunits orientation [206]; new generation of bNAbs to test the trimer conformation (PGT145 and PGT16) [207, 208] and the presence of hallmark epitopes; cryo-EM structure at 5.8Å [209] and crystal structure at 4.7Å [210] of resolution to visualize soluble Env trimers at atomic level.

5.1.1 Early generation trimers

Membrane-associated proteins such as Env are more difficult to produce and purify than secreted (i.e. soluble) ones. One of the first attempts to develop soluble trimers used constructs that encoded a truncated form of the gp41 subunit by adding a stop codon before

the transmembrane domain, resulting in soluble gp140 subunits containing the gp120 and gp140 ectodomains (gp140 $_{ECTO}$). Due to the non-covalent association between gp120 and gp41, these molecules were unstable and dissociated into gp120 monomers and gp41 $_{ECTO}$ (Earl, 1994). Later versions contained specific point mutations to eliminate the furin cleavage motif to generate covalent association of gp120 to gp41, but this approach generated heterogeneous preparations of molecules in different conformational states. The addition of heterologous trimerization domains such as GCN_4 and foldon, at the C-terminal region of gp41 improved the yield of the trimer fraction and allowed the production of sufficient amount of immunogens to perform animal studies (reviewed in [211]).

Such soluble gp140 foldon trimers (gp140-F) were first described in 2002 ([212]); they are composed of gp120, gp41$_{ECTO}$, a trimerization foldon domain derived from T4 bacteriophage fibritin and substitution mutations at the furin cleavage site (R/S 508, 511). Since then, their antigenic and immunogenic properties have been extensively characterized in small animals [22, 213-215] and in non-human primates (NHPs) [10, 11, 17, 51, 139, 216], providing a considerable amount of information about the induced polyclonal response. Immunogenicity studies in NHPs have allowed us to develop new tools, to dissect the polyclonal and to establish protocols to isolate monoclonal Abs from memB cells, as well as to define some fundamental characteristics of immune responses to Env vaccination in different compartments. In Paper I of this thesis, I used gp140-F trimers derived from the YU2 strain.

During Env vaccination, peripheral Env-specific IgG and memory B cell responses follow the same kinetics of induction and contraction ([51]; Paper I). This type of response is not unique to Env and is shared with other soluble, glycosylated recombinant proteins as we showed with influenza virus hemagglutinin (HA) [51]. Soluble YU2 gp140-F immunization readily induces plasma neutralizing titers of heterologous Tier 1 isolates and low neutralizing titers of the autologous Tier 2 YU2 strain. However, the specificities mediating the autologous Tier 2 activity were so far not defined at the MAb level ([10]; Paper I). By sorting single B cells for MAb isolation, we showed that the Tier 1 neutralizing activity is mediated by CD4bs and V3 specificities [11]. We also observed that the VH gene usage is similar in total and Env-specific IgG+ memB cell, and the response is highly polyclonal [10, 11, 41]. The level of SHM induced is between 2-14% [10, 11], although using more exact reference databases generated from the IgDiscover tool now offer more accurate estimations of SHM ([93]; Paper II).

Recently, new available tools such as bNAbs antigenic profiling and negative stain EM to evaluate the conformation of the gp140-F trimers made clear that these trimers have an open conformation that resembles Tier 1 viruses. This has shifted the focus to new generation trimers that better mimic native Tier 2 viruses, as described below.

5.1.2 New generation trimers

The intrinsic metastability of the native Env spike related to the non-covalent association of gp120 and gp41, as well as the propensity of gp41 to adopt post-fusion configurations,

represents a major challenge to design soluble mimics. In order to reduce this instability, the subunits were hold together by inserting two cysteines that form a disulfide link, and insertion of a point mutation (I559P) prevented the post-fusion structural changes, resulting in the SOSIP trimers [217, 218]. The first trimers using this SOSIP design were based on JRFL, a strain that intrinsically forms poor native-like soluble trimers [219], it was not until a truncation of $gp41_{ECTO}$ resulting in SOSIP.664, as well as *env* gene-screening programs that identified the intrinsic properties of clade A BG505 strain [220] that homogeneous trimers could be produced at higher yields. This resulted in the development of BG505 SOSIP.664 trimers that are considered a faithful mimic of the HIV-1 Env spike [221]. These trimers were successfully crystallized allowing the first definition of the trimer structure at a high level of resolution (Julien, 2013, Lyumkis 2013, Sanders, 2013). The SOSIP design applied to other strains was shown to result in mixture of homogeneous and heterogeneous trimers. Therefore, negative selection using nNAbs was shown to be an alternative strategy to significantly increase the yield of homogeneous trimers [219].

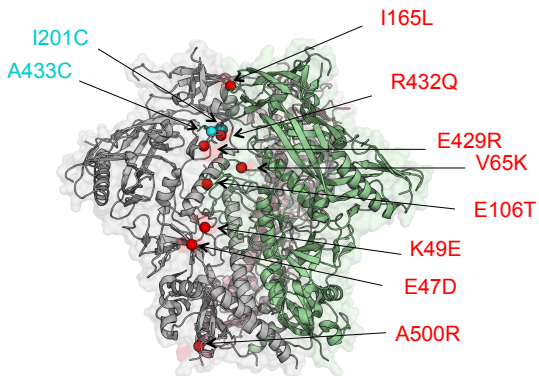

Figure 4. HIV-1 16055 NFL TD CC trimer. Ribbon representation of the 16055 NFL TD CC trimer structure derived from PDB entry 5CEZ, where the protomers of gp120 and gp41 are shown in gray and green. Spheres in red show the eight BG505 trimer derived (TD) residues that were substituted in 16055 NFL located in the gp120-gp41 interface, while light blue indicates the intraprotomer disulfide bond (I201C-A433C) located in the pre-ridging sheet. *Illustration by Javier Guenaga.*

Simultaneously, other efforts were made in order to generate faithful mimics of the HIV spike by using alternative approaches. In contrast to the SOSIP design, the native flexible linker (NFL) design maintains the gp120 and gp41 subunits covalently attached by replacing the furin cleavage motif REKR with a flexible linker (G4S) at the interface of these subunits, which together with the addition of I559P mutation results in cleavage-independent, native-like gp140 trimers [222]. The NFL trimers display antigenic and biochemical characteristics comparable to the SOSIP trimers [219, 222] and, similar to the SOSIP design, the NFL design derived from BG505 form highly homogeneous trimer preparations. However, further studies have shown that BG505 is an exception. Other strains, such as JRFL and 16055, form mixtures of well-ordered and less well-ordered trimers requiring further improvements to generate homogenous trimer preparations.

In order to improve the formation of homogeneous trimers, a careful inspection to the BG505 SOSIP crystal structure allowed the identification and transfer of residues implicated in trimer stability, referred as trimer-derived (TD) residues. For Clade C 16055 eight TD residues near the gp120-gp41 interface were transferred (47D, 49E, 65K, 106T, 165L, 429R, 432Q, and 500R) [207]. In addition, to avoid the conformational change induced in the trimer by the binding of primate CD4 [174], a disulfide bond (I201C-A433C) that locks the trimers in the pre-receptor bound conformation was introduced [207]. The resulting trimers were called NFL TD CC trimers, and their immunogenicity properties in NHP were evaluated in **Paper II**.

5.2 GERMLINE TARGETING

During the flourishing of bNAb isolations in 2009, it was reported that the putative unmutated germline versions of many bNAbs did not bind recombinant gp140 Env glycoproteins, while the mature version of the antibodies did [223]. Further studies with the now available bNAbs confirm this finding, suggesting that the soluble native-like trimers have features that do not allow them to interact with BCRs on naïve B cells, obstructing the induction of some bNAb precursors [34, 38, 46, 224]. This raises the question of how they were elicited in the first place. One possibility is that the right Env was not tested as it is difficult to know what Env was present in an infected individual early during infection, years before the Ab was isolated. Nevertheless, this has sparked an interest in the so-called germline-targeting approach to help focus the immune response on certain classes of bNAbs by activating specific germline-precursor B cells [225]. The design of immunogens capable of binding germline precursors requires an extensive knowledge of the mature bNAb features and epitopes as well as germline-encoded V, D, J segments, and has focused on Abs targeting the CD4bs, the trimer apex and the N332-supersite bNAbs (reviewed in [226]).

Engineered mice have been used to illustrate the concept of germline targeting, the first study of this kind was reported by Dosenovic, and showed that immunization with immunogens capable of germline targeting can activate the desired Ab precursors in mice strains with complete LC and partial HC reversion (residues outside of CDR3 reverted to germline) (GL_{HL}), and that different native-like immunogens are capable of promoting affinity maturation towards VRC01-class antibodies in another mice strain carrying a mature HC and reverted LC (Mut_{HC}) [227]. Since then many other mice strain have been develop to provide a more physiological setting to test the efficacy of germline target candidate immunogens [45, 228-230]. More recently a similar system was used to show that germline target immunogen follow by sequential boosting with progressively modified intermediates and wild-type immunogens plus a cocktail of Env-variable regions induce PGT121-like broadly neutralizing Abs in mice with partial HC reversion GL_{HL} and mature HC (Mut_{HC}) [45, 47].

6 MATERIALS AND METHODS

This section summarizes the main materials and methods used throughout the papers. For more detailed information, consult **Papers I-IV**.

6.1 RECOMBINANT ENVELOPE GLYCOPROTEINS

Two types of trimers were used in this thesis; the early generation YU2 gp140-F trimers (**Paper I**) and the newer generation 16055 NFL TD CC trimers (**Paper II**). The soluble gp140-F trimers consist of the gp120 subunit and the covalently linked ectodomain of gp41. These subunits are maintained as trimer by a heterologous trimerization domain (foldon, F) derived from T4 bacteriophage fibritin. To prevent gp120 disassociation from gp41, substitutions at the natural furin cleavage site (R/S 508, 511) were introduced [212].

The more highly engineered 16055 NFL TD CC trimers are composed of gp120 sequence and the ectodomain of gp41 covalently held together through a flexible linker (G4S) that replaces the native furin cleavage site (REKR; [222]). To increase their propensity to form trimers, mutations at eight trimer-derived (TD) residues were introduced (47D, 49E, 65K, 106T, 165L, 429R, 432Q, and 500R), deduced from the BG505 crystal structure, and to increase stability and to prevent CD4-mediated conformational changes, a disulfide pair was introduced at positions I201C-A433C in the bridging sheet region of gp120 [207]. Further, the I559P mutation [218] was introduced in gp41 to prevent trimer transition to the post-fusion state. In addition, an N-glycan naturally lacking at the 332N supersite in 16055 Env was introduced to restore the so-called "N-glycan supersite", a binding target for many broadly neutralizing antibodies [36]. The gp41 C-terminal region was truncated at residue D664 and fused in frame to a G4S linker and His6-8 capture tag. Production of these soluble trimers was previously described [174, 207]. In brief, trimers constructs were transiently transfected into 293F cells using 293-fectin and cell culture supernatants containing the expressed Env proteins were collected 5-6 days post-transfection. The Env glycoproteins were purified by Galanthus nivalis lectin-agarose affinity chromatography followed by size-exclusion chromatography (SEC) to isolate the trimer-containing fractions.

6.2 CONJUGATION OF THE TRIMERS TO THE LIPOSOMES

Liposome preparation and well-ordered trimer conjugation has been described previously [231]. In brief, the liposomes are composed of 50% DGPC (1,2-distearoyl-sn-glyero-3-phosphocholine), 36% cholesterol and 4% 1,2-dio-leoyl-sn-glycero-3-((N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl) (nickel salt) DGS-NTA(Nickel). Inclusion of the charged nickel containing head group allows chelation of the histidine-tagged 16055 NFL TD trimers. The liposomes were formed by the solubilizing the three lipid components in chloroform, drying the lipids on to a glass surface, resuspension in aqueous buffer (PBS) to create lipid micelles. Following sonication to create single monolayers, the liposomes were extruded through a series of filters to obtain particles of approximately 100 nM in diameter as confirmed by DLS (dynamic light scattering). Conjugation of the His-tagged trimers was

accomplished by incubation of approximately 2.2 mg of (excess) trimer with 500 µl of liposomes for 2 hours (hrs) at room temperature (RT). The unbound trimers were resolved from the trimer-conjugated liposomes by passage over a Superdex 200 sizing column. The trimer--conjugated liposomes were pooled and stored at 4ºC before use.

6.3 ANIMALS

Rhesus macaques (Macaca Mulatta) of Chinese origin were immunized and sampled for **Papers I** and **II**. Since samples collected from previous studies were used to conduct assays shown in **Papers III** and **IV**, no additional animals were used for these papers. Housing and care procedures of the animals were done at the Astrid Fagraeus Laboratory animal facility at Karolinska Institutet according to the provisions and general guidelines of the Swedish Board of Agriculture. All animals were habituated to the housing conditions for around 6 weeks before beginning the experimental procedures. They were kept in pairs in $4m^3$ cages with enriched environment and they were confirmed negative for SIV, simian lymphotropic virus and simian retrovirus type D.

6.4 IMMUNIZATION AND SAMPLING

The formulation for each inoculation was composed of 100µg of trimers and 75µg of Matrix-MTM. Where indicated, CpG ODN 2395 (500 ug) was added. Animals were inoculated two (**Paper I**) or three (**Paper II**) times with a short interval, and once (**Paper I**) or twice (**Paper II**) with a long interval. All inoculations were given intramuscularly (i.m.) in a total volume of 1 ml, divided equally between the left and right leg. Immunizations and blood samplings were done under sedation with 10 mg/kg ketamine i.m. An additional dose of 0.5mg/kg Xylazine was given i.m. when the animals were sampled for BM and lymph nodes (LNs) to induce relaxation and analgesia. Blood and BM were collected in 9ml vacutainer tubes with EDTA, cells isolation was performed with Ficoll-Hypaque, and after red cells lysis, the cells were extensively washed with PBS before freezing in fetal bovine serum with 10% DMSO.

6.5 HIV-1 PSEUDOVIRUS NEUTRALIZATION ASSAY

Neutralizing Ab activity by plasma or purified MAbs was measured in **Papers I** and **II** using the single round TZM-bl assay [232]. This assay measures the antibodies capacity to block infection of panels of viruses pseudotyped with different Env glycoproteins using reduction of luciferase activity as the read-out [18]. Pseudoviruses are generated by co-transfection of 293T cells with two plasmids, one encoding the HIV-1 Env of interest, and the other encoding HIV-1 gag-pol resulting in single round infectious viruses that cannot further replicate due to a lack of packaging signal in the construct encoding Env. TZM-bl cells are HeLa cells modified to stably express the HIV-1 surface receptors CD4, CCR5 and CXCR4 allowing infection of most HIV-1 viruses, as well as to encode a luciferase reporter gene under transcriptional control of the HIV-1 LTR. Luciferase is expressed when the TZM-bl cells are infected through Tat-mediated activation of the LTR-luciferase gene. In the neutralization assay, pseudoviruses to be tested are pre-incubated with a dilution series of plasma or MAbs and incubated with the TMZ-bl cells. Neutralization activity is reported as

plasma Inhibitory Dilution 50 (ID50), which is the reciprocal of the plasma dilution resulting in 50% inhibition of virus infectivity or, for purified MAbs, as inhibitory concentration 50 (IC50) for the concentration of MAb resulting in 50% inhibition of virus infectivity.

The Env glycoproteins used to generate pseudoviruses in this assay have been categorized according to their neutralization resistance using well-characterized MAbs and IgG fractions from HIV-1 infected individuals [232]. Tier 1 viruses (classified as tier 1A and tier 1B) are sensitive to neutralization and therefore are not representative of circulating HIV-1 strains. They are lab-adapted or in some cases natural strains associated with an open conformation, making them sensitive to commonly elicited Ab specificities such as against the V3 region. Tier 2 viruses are neutralization resistant and represent primary circulating HIV-1 strains. Most viruses belong to this tier. Tier 3 viruses represent a minor portion of viruses with exceptional neutralization-resistant phenotypes.

6.6 B CELL ELISPOT ASSAY

The protocols to evaluate antigen-specific memB cells and PCs frequencies in rhesus macaques were previously standardized by our group [139]. In brief, antigen-specific memory B cell frequencies are determined after their differentiation into antibody-secreting cells (ASCs). This is done by stimulating total PBMC with a cocktail containing CpG-ODN-10103, pokeweed mitogen (PWM) and Staphylococcus aureus Cowan strain (SAC) for 4 days at 37°C with 5% CO2 in 48-well plates. After the culture the stimulated cells are extensively washed and transferred into ELISpot plates previously coated with anti-IgG. The detection of antigen-specific PCs does not require stimulation and bone marrow cells can be plated directly into previously coated ELISpot plates. After overnight (ON) incubation on the ELISpot plate, the cells are washed and total and antigen-specific IgG can be detected by the addition of biotinylated anti-IgG or Env probe, respectively. The plates are then washed and incubated with streptavidin-ALP followed by BCIP/NBT substrate. Spots that correspond to Ab-secreting cells (ASCs) are then counted. The frequencies of total and specific ASCs per million cultured PBMCs or plated BM cells (ASC/10^6 cells) are calculated, as well as the frequency of antigen-specific cells out of the total ASCs number.

6.7 FLOW CYTOMETRY

LSRII and FACS-Aria flow cytometry instruments located at the MTC FACS Facility were used for multiple purposes in this thesis: to evaluate intracellular cytokine production of Env-specific T cells in **Paper I**, to sort antigen-specific memory B cells in **Paper II** and to phenotype plasma cells in **Paper IV**.

To evaluate the frequency of Env-specific T cells and cytokine production in **Paper I**, we used a protocol previously described [216], where PBMCs were stimulated for 6 hour with a pool of Env peptides together with anti-CD28, anti-CD49d and Brefeldin A to activate and stop transport of cytokines from the endoplasmic reticulum to the Golgi apparatus, respectively. Cell viability was determined with a Live/Dead fixable dye followed by surface stain with anti-CD4 and anti-CD8. This was follow by permeabilization and intracellular

stain with anti-CD3, IL-2, IFN-γ and TNF-α. All Ab stains were added in Facs buffer (PBS supplemented with 2% FBS) and incubated for 15min on ice. Surface stain washes were performed with FACS buffer, while intracellular stain washes were done using perm wash buffer.

In **Paper II**, we sorted Env-specific memB cells based on a previously described staining panel [10]. Env-specific memB cells were defined as CD3-CD14-CD20+CD27+IgG+Env+. Fresh PBMCs from animal D11 were collected and stained for cell viability with a Live/Dead fixable stain follow by the surface stain with the panel describe before. Stained cells were single cell sorted into 96-well plates containing lysis buffer [233], and plates were immediately spun and frozen on dry ice prior to storage at -80 °C.

Phenotypic characterization of rhesus PCs in **Paper IV** was done by first using FACS to test the staining pattern of new markers and then to evaluate the functionality of the PCs by ELISpot based upon the expression of the new markers described. In brief, rhesus BM PCs were thawed and stained for cell viability with a Live/Dead fixable stain followed by a surface stain with CD3, CD20, and CD138 together with the new markers CD31, CD49d and CD98. To test the functionality of the PCs by ELISpot, freshly collected rhesus BM was stained for cell viability followed by surface staining as described above. CD3-CD20-CD138+CD31+ BM cells were sorted into RPMI media supplemented with 10% FBS and kept on ice until diluted and added to ELISpot plates previously coated with anti-IgG.

6.8 SINGLE-B CELL CLONING AND ANTIBODY EXPRESSION

Sundling *et al* have previously described a protocol for single-cell cloning and antibody expression from rhesus macaque memB cells [10]. In brief, reverse transcription was performed in the single cell sorted 96-well plate using random hexamers and Superscript III reverse transcriptase. This was then followed by multiplex nested PCR of individual plates for IgH, Igκ and Igλ to amplify H- and L- chain V(D)J segments, respectively. The multiplex primer mixes consisted of 5' primers mixes covering the V-segment families and one 3' primer located in the IgG constant region [10]. To determine V(D)J usage, positive wells from the nested PCR were sent for sequencing. High fidelity cloning PCR with customized primers was done to add restriction sites for cloning the VDJ region into expression vectors. Cloning PCR products were evaluated on agarose gel and then purified, followed by enzymatic digestion and ligation of the H- and L-chain cloning products into expression vectors containing human Igγ1-H, Igκ1-L or Igλ2-L constant regions, the ligation mix was added to ultracompetent E.coli by heat shock at 42°C for 45 sec and then set to grow on agar plates containing ampicillin ON. To confirm that bacterial colonies contained plasmids with the correct insert size, PCR colony screen was done and positive inserts were sent for sequencing to verify the original sequence obtained after the nested PCR. When the sequence was 100% consistent, matching IgH and IgL chain vectors were co-transfected into HEK 293-F cells using Freestyle MAX reagent. ELISA was done to evaluate the presence of total and Env-specific Abs in the supernatants. Cultures positive for functional Env-specific Abs

were harvested 7 days after transfection and purified using Protein G Sepharose columns. All purified recombinant MAbs were further analyzed by SDS-PAGE under reducing condition.

7 RESULTS AND DISCUSSION

7.1 KINETICS OF B CELL RESPONSES UPON SOLUBLE ENV TRIMER IMMUNIZATION

Like most purified recombinant proteins, Env does not elicit appreciable Ab responses in the absence of adjuvant [234, 235]. Matrix-M is a relatively potent adjuvant that stimulates both T- and B-cell immunity as shown both in experimental animals [73, 74] and in humans [75]. We have used Matrix-M to elicit high antibody titers to soluble Env immunogens in several NHP immunogenicity studies ([17, 51, 139, 216] Paper I and II). However, as for previous Env immunization studies [236], we have shown that Env-specific plasma IgG titers declines relatively fast in the absence of boosting [51, 139], suggesting that most of the antigen-specific IgG is produced by short-lived PCs.

As part of this thesis, I therefore evaluated whether the addition of TLR-9 agonist (**Paper I**) or the use of liposomes for Env display (**Paper II**), both in the presence of Matrix-M, would impact the magnitude or the durability of the response over that seen with Matrix-M. Consistent with our previous reports, Env-specific binding titers reached peak titers after the second immunization and these responses were not further boosted after additional injections. This was similar whether Env and Matrix-M was used alone or in the presence of TLR9 stimulation (**Paper I**), or liposome display (**Paper II**). The contraction of the response was also similar and corresponds to the Ab half-life. Thus, TLR9 stimulation or liposome display did not detectably impact on the kinetics of the response observed with Env and Matrix-M alone.

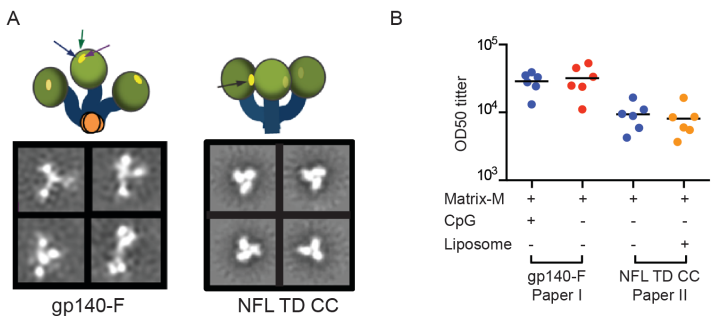

Figure 5. Early and new generation of HIV-1 Env trimers and their elicited Ab binding titers. (A) Representation and negative stain EM of gp140-F trimers (left) and NFL TD CC trimers (right). **(B)** IgG Env-specific titers were measured by ELISA two weeks after the second immunization. Each dot represent one animal and the lines represent the mean OD50 binding titer. Andrew Ward and Natalia de Val performed the negative stain EM.

Although the kinetics followed the same pattern in **Paper I and II**, the overall magnitude of the responses was lower in **Paper II** (Figure 5) mainly due to the different immunogens used in the studies. In **Paper I**, we immunized with gp140-F soluble trimers, while in **Paper II**, we used the new generation of soluble NFL TD CC that are more stable and homogeneous, and

were designed to better occlude non-broad neutralizing epitopes, consistent with the lower magnitude of the induced Ab response.

B cell responses in the periphery were evaluated in **Paper I**. Total IgG memB cell frequencies in peripheral blood were constant during the immunization regimen, corresponding to 0.5% of the cells. Of these, approximately 10% were Env-specific memB cells at peak responses and Env-specific memB cells followed the same kinetics as the plasma Ab response. This suggests that most Env-specific circulating Abs arise from short-lived PCs that differentiated upon antigen re-exposure of antigen-specific memB cells [11]. The rectal and vaginal mucosal IgG Env-specific Ab levels also followed the same kinetics as the plasma IgG titers, indicating that systemic immunizations generate antigen-specific Ab that disseminate to the mucosa through transcytosis, which could play an important role in containing the mucosal infection as has been shown for HPV [237, 238].

B cell responses in the BM were evaluated in **Paper I** and **III**. In **Paper III** we showed that longitudinal BM aspirations do not have a detrimental effect on the frequency of total BM PCs and that the frequency of total PCs was proportional to the animal age, with older animal displaying higher frequencies. In **Paper I**, BM PCs in rhesus macaques remained constant during the course of the immunization regimen and represented 0.2% of BM cells. Of these around 1% were Env-specific IgG during the short interval and before the long-term interval boost, consistent with our previous reports [51, 139]. However, the frequency of Env-specific IgG PCs after the long-term interval boost described in Paper I was significantly increased to 8% of the total PCs. It will be of interest to investigate which Ab specificities are recruited to the BM compartment, as well as to obtain a better general understanding of the dynamics between the BM and the peripheral immune compartments.

7.2 PHENOTYPIC CHARACTERIZATION OF RHESUS BONE MARROW PLASMA CELLS

In **Paper IV**, we characterized rhesus macaque PCs in the BM compartment by evaluating markers previously used to define human and mouse BM cells such as CD49d [239], CD98 [240] and CD31 [241, 242]. Staining of CD3- CD20- BM cells with these new markers together with CD138 allowed a clear distinction of double positive cells composed of functional IgG-, IgA- and IgM-secreting BM cells. Functionality was confirmed using B-cell ELISpot analyses of sorted cell populations. The co-expression of CD138 together with CD31, CD48d and CD98 allowed differentiation of BM PCs from PCs present in peripheral blood. We also found that both human and rhesus PCs similarly co-express CD138 and CD31, and rhesus PCs secrete the same proportion of isotypes with IgG and IgA being most abundant followed by IgM as has been described for humans [243, 244]. The capacity to stain functional BM-resident PCs opens up possibilities to sort these cells for MAb isolation and additional analyses at the single cell level.

7.3 MODULATION OF NEUTRALIZATION ACTIVITY

Measurement of the Ab neutralization levels in plasma is the main end-point for many human vaccines. We have also evaluated neutralization activities in plasma as a first but not unique read out of the response in **Papers I** and **II**, both at the polyclonal and monoclonal levels.

7.3.1 Plasma neutralizing Ab activity

Our findings regarding HIV-1 neutralizing Ab activity in plasma can be briefly summarized as follows:

Paper I, a) plasma neutralizing Ab activity was not modulated by the addition of TLR-9 agonist, b) the neutralizing Ab activity was mainly directed against Tier 1 viruses with low titer activity also detected against the autologous Tier 2 virus and c) induction of CoRbs-directed Abs was evident as shown using an HIV-2-based assay in the presence or absence of soluble CD4.

Paper II, a) particulate display of trimers on the surface of the liposomes induced similar Tier 1 neutralizing Ab levels as soluble trimers, b) neutralizing Ab activity against the Tier 2 16055 autologous virus was significantly increased and more consistent in the liposome group compared to the soluble group and c) CoRbs-directed neutralizing Ab activity was not detected, likely as a result of the disulfide bond that impairs CD4-induced conformational changes as described above.

7.3.2 Monoclonal antibodies

An improved understanding of the fine specificities mediating neutralization of Tier 2 viruses even in the absence of neutralization breadth is of great interest for the vaccine development. This requires cloning of antigen-specific MAbs. Therefore, in **Paper II**, we isolated a panel of twenty MAbs from animal D11 immunized with liposome-displayed trimers. D11 was chosen as it displayed the highest titers autologous neutralizing Ab activity after the third immunization suggesting that this was a good source for MAbs isolation. Of the 20 MAbs, 18 were shown to be Env-specific and these were categorized according to their binding capacities to monomeric gp120 and to a V3 peptide. The MAbs were also examined for their neutralizing activity against Tier 1 and Tier 2 viruses. MAbs with autologous Tier 2 neutralization represented 15% of the specificities cloned and these MAbs were subjected to further characterization. Through close collaborations with the Wyatt laboratory, we mapped the epitopes of these Tier 2 neutralizing Abs and were able to show that they target V2 region of the HIV-1 Env trimer and that they do so by a lateral approach towards the apex. One previous study using BG505 trimers reported elicitation of autologous Tier 2 neutralizing Abs in NHPs; however, the specificities mediating this activity were not defined at the MAb level [21]. Isolation of MAbs from rabbits immunized with BG505 trimers showed that the autologous Tier 2 neutralization targets a different region of Env [23], suggesting that different Env trimers display different sites of vulnerability that explains why autologous but not heterologous neutralization is achieved. The objective for the field for the next few years

will be to define if more conserved epitopes can be targeted by vaccination, perhaps with well-designed heterologous prime-boost regiments to target the response on relevant neutralization targets with the ultimate aim to improve the breadth of neutralization achieved.

7.4 AFFINITY MATURATION

The progressive increase of serum Ab affinity over time, results from the affinity maturation that involves the generation of a repertoire of mutated clonal variants and the positive selection of high affinity clones. We have used the avidity index as an indicator of Ab maturation [139]. Similar to what we have reported previously, in **Paper I** the long-term interval seems to have a positive impact in the functional avidity in the absence of boosting (Figure 6), which implies that the affinity maturation was taking place in ongoing GC reactions was possible also in the absence of boost, consistent with detection of GC reaction up to 120 days after boost [245]. In **Paper II** we also used a long-term interval, and we observed boosting in the neutralizing antibody response following the 3rd (most animals) and 4th (some animals) immunization, but not by the 5th immunization, in addition the autologous tier-2 MAbs display high affinity binding (nM levels) that correlates with SHM (8-13% aa level) already after the third immunization, these findings support the idea that by the 5th immunization autologous tier-2 neutralizing antibodies have reached an affinity ceiling [43], consistent with what has been reported before with tetanus toxoid immunization [246].

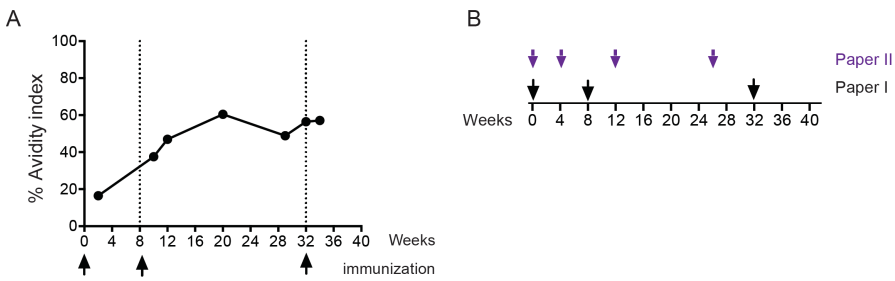

Figure 6. Long-term interval functional avidity increase. (A) Avidity index was used as an indication of antibody maturation in Paper I; plasma from immunized macaques was evaluated in a NaSCN wash assay. Avidity index was calculated as: (OD50 titer NaSCN/PBS)x100. **(B)** Long-term immunization scheme used in Paper I and Paper II

7.5 PARTICULATE DISPLAY

The mechanism by which multimeric display of trimers on liposomes induces higher and more consistent autologous Tier 2 neutralization has not been investigated. In mice the trimer-arrayed liposomes are more efficient at activating Env-specific B cells *in vitro* and at inducing GC B cells *in vivo* than the soluble trimers [231]. In our studies in NHPs, we reported superior GC responses after immunization with liposome-displayed trimers (**Paper II**) leading us to hypothesize that multimeric display of Env enhances B-cell activation by allowing a greater number of low affinity B-cell clonotypes including those with neutralizing potential to enter the GC reaction. Our data suggest that proper activation of both GC B cells

and Tfh cells in the early immune response is essential and is better achieved by the liposome multimeric display. That the autologous Tier 2 neutralizing titers do not markedly increase with further boosting suggests that these Abs have reached an affinity ceiling. To formally investigate the level of SHM at different time points during the immunization regimen would require sequencing V genes from large numbers from Env-specific memB cells and is the objective of other ongoing work in our group.

Particulate display of antigen may be a general way to improve immune responses. For example, it has been reported that virus-like nanoparticles efficiently activate B cells [46] and plasma membrane sheets better trigger BCR signaling of GC B cells [119]. Furthermore, the only two recombinant protein-based vaccines that are licensed so far, the hepatitis B vaccine (HBV) and the human papilloma virus (HPV) vaccine are based on virus-like particles that display the antigen in a multimeric manner [247, 248]. The generation of HIV-1 VLPs that express functional spikes is challenging due to the intrinsic characteristics of Env such as low density in the virions and instability; however, recent reports demonstrate some progress in this regard [249].

8 CONCLUDING REMARKS AND FUTURE DIRECTIONS

The goal of vaccination is to induce protective immune responses that persist over time, so also for HIV-1. To achieve this, the evaluation of novel vaccine strategies that promote Ab affinity maturation and durability of Abs targeting relevant epitopes is needed. A better understanding on how to generate stable native-like soluble HIV-1 Env trimers that expose bNAb but not nNAb epitopes was obtained only in the last couple of years. In the work presented in this thesis, we used early and new generation soluble HIV-1 Env trimers to characterize B cell responses in immunized rhesus macaques, a highly relevant animal model of human biology. In **Paper I** and **II**, we evaluate immune-modulatory molecules and novel presentation platforms, while in **Paper III** and **IV** we assess and better characterize the BM plasma cell, a compartment important for the durability of the response.

In **Paper I**, we performed an immunization and SHIV challenge experiment to investigate if the addition of a TLR-9 agonist to early generation Env trimers formulated in Matrix-M would qualitatively or quantitatively alter the effect of vaccination. We observed similar B cell and neutralizing antibody responses as well as comparable peripheral T cell responses and control of viremia after challenge. Thus, TLR-9 stimulation did not enhance the response induced by Env in Matrix-M, suggesting that Matrix-M can be used as a stand-alone adjuvant. **In Paper II**, we evaluated the immunogenicity of new generation well-ordered Env trimers. We asked if Env conjugation to liposomes for multi-valent display would offer a benefit over administration of soluble trimers in the presence of Matrix-M. Our results demonstrate that while the induction and magnitude of Env-binding antibodies in the periphery was similar between the two platforms, early germinal center responses and autologous Tier 2 neutralization were superior after immunization with the liposome-conjugated trimers. Dissection of the response by isolation of monoclonal antibodies (MAbs) revealed that autologous Tier 2 neutralizing MAbs target a variable V2 region in the Env trimer apex using a lateral binding approach that permits access to the epitope through the glycan shield. This information can be used to modulate the design of trimers to focus the response on more conserved determinants in the V2 apex. Future work will evaluate more stable ways to conjugate the trimers to the liposomes through covalent coupling.

Taking into account the poor understanding of the bone marrow (BM) antibody compartment, in **Paper III**, we determined the frequencies of total functional plasma cells in rhesus BM, and demonstrated that frequencies of total plasma cells correlate with the age of the animals and are not affected by longitudinal BM sampling, which offers a basis for further studies of vaccine-induced responses in this compartment. In **Paper IV**, we identified cell markers that better characterize functional BM plasma cells in rhesus macaques. We established that double positive CD138 and CD31 cells constitute the functional BM plasma cells that constitutively secrete IgG, IgA and IgM. These markers also stained human BM plasma cells. The ability to better identify functional BM plasma cells allows sorting of these cells for MAb isolation and molecular analyses at the single cell level to further improve our understanding of the dynamics and characteristics of plasma cells in this compartment.

In conclusion, this thesis offers new information about vaccine regimens to induce antibody responses against HIV-1 Env trimers, including evaluation of adjuvants and presentation platforms using early and new generation Env trimers (**Paper I and II**). This thesis also establishes new methodology that can be used to further investigate vaccine-induced B cell responses in the BM compartment (**Paper III and IV**). Each of these studies has relevance for our basic understanding of immunology as well as for practical vaccine development.

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